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TITLE: Prevention of Post-Radiotherapy Failure in Prostate
Cancer by Vitamin D

PRINCIPAL INVESTIGATOR: Srinivasan Vijayakumar, M.D.

CONTRACTING ORGANIZATION: University of California
Davis, CA 95616-8679

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INTRODUCTION

We plan to conduct a phase I/II safety/chemoprevention study to determine whether taking a non-toxic Vitamin D analog, $1\alpha(\text{OH})\text{D}_5$ (D5), can safely delay prostate cancer recurrence when administered after radiation therapy (RT). The newly synthesized analog $1\alpha(\text{OH})\text{D}_5$ (1α -Hydroxy-24-ethyl-cholecalciferol) has shown anti-tumor activity at non-hypercalcemic concentrations in animals. Based on their preliminary research, investigators in this study believe D5 can be given in effective doses without causing harmful side effects. Forty randomized patients will receive either D5 or placebo, 12-60 months after completion of RT (20 patients/arm). During the study patients will be closely monitored for hypercalcemia as well as other potential toxicities. At the end of the study, subjects will receive final laboratory and clinical evaluations and undergo a prostate biopsy. Study endpoints include differences between study groups in drug tolerance and compliance, toxicity, quality of life, biomarker presence and proportion of patients developing PSA-based biochemical failure or clinical failure. Biopsies will be evaluated for selective markers indicating any benefit of D5 in decreasing the recurrence of prostate cancer and also for any differences between the groups in terms of expressed intermediate molecular biomarkers. Patients will continue to be followed for any clinical recurrences or toxicity as part of their usual cancer care.

BODY: WORK DONE AND ACCOMPLISHMENTS

Grant Transfer

In February 2004, the grant was finally and officially transferred from the University of Illinois at Chicago (UIC) to the University of California, Davis (UCD), a necessary step in allowing us to conduct the study once we obtain IRB approval at UCD and DOD approval.

Completion of Clinical Protocol and Approval by UC Davis IRB

Our principal accomplishment during the past year has been finalizing the clinical protocol for the study with D5 and securing the approval, with pending minor revisions, by the UC Davis IRB for the clinical trial (See Appendix 6). On March 8, 2004 the UC Davis IRB met and approved the protocol, pending minor revisions. We are making those revisions now (mostly wording changes) and will soon resubmit the protocol to the IRB Committee Chair for final approval.

The development of the clinical protocol began by taking into account the critique of the protocol made by the UIC Cancer Center Protocol Review Committee in July 2002. While at UIC, Dr. Vijayakumar brought the protocol to about 80% completion. He had set up an Executive Committee to prepare the protocol, and they met several times to design the study. (Minutes were submitted to the DOD previously.)

Further fine-tuning occurred at UC Davis. In 2003, Dr. Vijayakumar shared the protocol with UCD Radiation Oncology faculty at regular faculty meetings, seeking their input on how to improve the protocol and incorporating their suggestions. Attendees at these meetings were Radiation Oncologists Dr. Allan Chen, Dr. Rachel Chou, Dr. Zelanna Goldberg, Dr. Samir Narayan and Dr. Janice Ryu, and Physicists Dr. Julian Perks, Dr. Robin Stern, and Dr. Claus

Yang. In addition, over several months in the fall of 2003, Dr. Vijayakumar consulted extensively with the statistician for the UCD Cancer Center, Dr. Laurel Beckett, to confirm and modify the study design. Dr. Vijayakumar also recruited other investigators for the protocol, especially clinical faculty who will be enrolling patients in the trial, and assembled the rest of his team for the study (Clinical Research Associates, consultants).

In October 2003, Dr. Vijayakumar made a presentation to discuss the protocol with several UCD Cancer Center faculty. At the meeting was the director of the Cancer Center, Dr. Ralph DeVere White (Urology), as well as Dr. Samir Narayan (Radiation Oncology), Dr. Paul Gumerlock (Hematology & Oncology), Dr. Rajendra Mehta--via speaker phone (Surgical Oncology, UIC), Dr. William Hall (Radiation Oncology), and Phil Boerner (Writer, Radiation Oncology). As a result of this meeting, several important modifications were made to the protocol, including adjusting eligibility criteria, study endpoints, and having a data and safety monitoring committee review the study periodically once it commences.

Incorporation of Pre-review from DOD

Before submitting the updated protocol to the UC Davis Cancer Center Scientific Review Committee, Dr. Vijayakumar wanted to have input from the DOD's pre-review. In November 2003, Dr. Vijayakumar received the DOD pre-review of the Vitamin D5 study, and incorporated the valuable suggestions made there into the protocol.

Submission and Approval from UCD Cancer Center Scientific Review Committee

In December 2003, Dr. Vijayakumar made a presentation to the UCD Cancer Center Scientific Review Committee and subsequently this committee approved the D5 protocol (see Appendices 1 and 2). (This committee's approval is required prior to submitting a protocol to the UCD IRB.) On the advice of this committee, we added a "Treatment Plan" section to the protocol.

Submission to UCD IRB

On February 19, 2004, the D5 protocol was submitted to the UC Davis IRB (see Appendix 5). The protocol was approved, pending minor revisions, on March 8, 2004. When we make the minor revisions and obtain final IRB approval, we will submit the protocol to the DOD for approval.

Comments on First Annual Report (March 2003)

We wish to comment on the critique of our first Annual Report, in order to clarify some issues. The statements from the critique are in italics, and our responses follow.

1. *"To evaluate the efficacy of this analog, the Principal Investigator (PI) will evaluate 1 α (OH)D5 in prostate cancer cell lines."*

The DOD did not fund this part of the research, so that will not be a part of what we accomplish with this grant.

2. *"The PI should note that an abstract and more than one subject term are required in form 298. In addition, a list of key research accomplishments, reportable outcomes, and references are required elements of the annual report."*

All of these required elements are included in our March 2004 report.

3. *Data presented in the first annual report showed that Tasks 1-3 were not initiated.*

The first tasks in the original plan called for basic science research. That part of the proposal was not approved by the DOD and so we could not accomplish them as part of our work, at least not with grant funding. We reported the basic research we had conducted, even though it was with other funding. This basic research was important in regard to the clinical trial to continue to demonstrate the safety and efficacy of D5.

4. *"the PI is no longer at the University of Illinois, and that the grant transfer request is in process"*

The March 2003 report was written following a year of transition for the D5 project. The PI, Dr. Vijayakumar, left the University of Illinois at Chicago (UIC) in the fall of 2002 and started his new position as chairman of the Department of Radiation Oncology at U.C. Davis in November 2002. This precipitated a process of transferring the DOD grant from UIC to U.C. Davis, a process which we could not control and which only recently became complete—the transfer became official in February 2004.

5. *"all of the work reported here deals with basic scientific research, which was deleted from the project per the scientific review panel"*

A phase I trial was part of the original application to the funding agency, the U.S. Army Research and Materiel Command (USAMC). However, the USAMC funded only the clinical trial. While we were pleased with this in that it allowed us to move more rapidly in addressing an important problem—namely, prevention of recurrence of prostate cancer in high-risk patients treated by radiation—it also made it impossible to achieve the originally stated laboratory research goals.

6. *"the clinical protocol was not approved" (at the University of Illinois at Chicago)*

We wish to clarify that the UIC's IRB never considered the D5 protocol; because the principal investigator left UIC, his protocol did not get as far as that institution's IRB. Rather, the protocol was considered by UIC's Cancer Center Protocol Review Committee. We took the Cancer Center Protocol Review Committee's comments into consideration when rewriting the protocol for submission to the UC Davis Cancer Center Scientific Review Committee, which is UC Davis's equivalent of UIC's Cancer Center Protocol Review Committee. We have since earned approval for the protocol at the UCD Cancer Center Scientific Review Committee (Appendix 2) and taken it to the UCD IRB (Appendices 5 & 6).

7. *"no progress has been made on the tasks being funded by the Department of Defense"*

The DOD is funding a clinical trial with D5, and we have been undertaking tasks which will bring about this clinical trial. These tasks have been either preliminary laboratory studies with D5, or preparation of the protocol for the clinical trial. Therefore, we feel that progress has been made on the tasks being funded by the DOD.

The March 2003 annual report described some of the initial laboratory studies that Dr. Vijayakumar and his colleagues had completed with D5. These had been planned based on the original proposal to the DOD, which featured a two-phase study that consisted of a Phase I experimental component and a Phase II clinical trial. However, the DOD felt that D5 was so promising as a chemopreventive agent that it approved proceeding with the clinical (second) phase as soon as practical.

In the meantime, however, at UIC the Executive Committee approved some D5 laboratory studies and the use of minimal DOD funding as the group waited for clinical protocol development to occur. The Executive Committee also planned other D5 laboratory investigations, not funded by the DOD grant for D5. Both types of research were reported in March 2003. In fact, only about \$14,000 has been spent from the DOD grant money for D5; of that, about \$5,000 has been used for laboratory studies. At the time we made those expenditures, we misunderstood that it was not approved to use some grant money for laboratory studies relating to D5, something we had done only after UIC Executive Committee approval.

Since joining UC Davis, Dr. Vijayakumar and his colleagues have not charged any expenses against the DOD funding, despite devoting a substantial amount of time to the development of this protocol. Dr. Vijayakumar has decided to use DOD funding only after all the approvals have been made to the D5 protocol and his team is ready to begin the clinical trial, in order to enable the successful completion of the clinical trial.

8. *"from the Statement of Work (SOW) provided, it appears that the entire study should be completed in 1 year"*

We wish to clarify that ours is a 3-year project, and always has been. The clinical trial itself will take two years, and there is preparatory and follow-up work to the clinical trial (recruiting patients, their one-month trial period with a placebo, etc.). There were basic science research goals for a first year, but that was not the entire study (and that portion of the grant was dropped anyway).

KEY RESEARCH ACCOMPLISHMENTS

The focus of the past year has been on securing approvals for the clinical trial with D5. However, the principal investigator and his colleagues have published research findings on D5 over the past year. This research is preparatory to conducting the D5 clinical trial and has not necessarily been funded by the DOD grant to Dr. Vijayakumar.

During the past year (see Appendix 4 for articles cited):

1. Dr. Vijayakumar and his colleagues showed that D5 has cell-differentiating and antiproliferative actions in breast cancer cells, and that this analog is tolerated at a very high concentration without causing severe toxicity. These results suggested that D5 may be a valuable therapeutic agent for prostate cancer treatment. This work was supported in part by the D5 grant (Babbar 2003).
2. The PI's colleagues studied the effect of a dietary supplement of D5 on a herceptin-resistant human breast carcinoma cell line in athymic mice. In vivo, animals receiving a D5-supplemented diet had significantly smaller tumors after 14 days of diet compared to animals in the control group. Tumors were excised and examined. Results suggested that D5 mediates its action by regulating the cell cycle-associated proteins in herceptin-resistant breast cancer cells and may be considered for its therapeutic value (Graves 2003).
3. The PI's colleagues wrote a review article summarizing their research with D5 and its application in breast cancer prevention and therapy. D5 has been shown to inhibit development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture model. Moreover, the inhibitory effect was more significant if D5 was present during the promotional phase of the lesion development. The growth inhibitory effect of D5 was also manifested in several breast cancer cell lines. Breast cancer cell lines that responded to D5 were vitamin D receptor positive (VDR+). Breast cancer cell lines that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis. Their findings imply a differential effect of D5 on ER+ vs. ER- cells (Hussain 2003).
4. The PI's colleagues showed that D5 was tolerated by rats and mice at a much higher dose than D3 (another vitamin D analog, which has been studied more extensively and is more likely to cause hypercalcemia). This property makes D5 a prime candidate for chemoprevention studies. Their studies collectively indicate that D5 selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. The growth inhibitory effects of D5 were observed in vitamin D receptor positive (VDR+) breast cancer cells, but not in highly metastatic VDR- breast cancer cells, suggesting that D5 action may be mediated, in part, by VDR (Mehta 2003).
5. The PI's colleagues evaluated D5 for its chemopreventive activity on colon carcinogenesis. Specifically, they investigated the effects of D5 on multiplicity and size of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in mice. D5 significantly reduced multiplicity of ACF, resulting in 95% suppression of ACF formation. Ongoing studies are being conducted to examine the effects of D5 during the initiation and post-initiation stages of colon carcinogenesis. Also, the mechanism of action for D5 was investigated in several human colon cancer cell lines. Western blot analyses showed that treatment of D5 resulted in down-regulation of E2F1 and Cyclin B expression following 3 and 5 days of treatment with D5, respectively. These results lend support to the potential use of D5 as a candidate for chemoprevention and treatment of colon cancer (Murillo 2003).

6. The PI's colleagues examined whether D5 specifically delivered to breast cancer cells could have any therapeutic effect. D5 was linked to Her-2 antibody. D5-Her-2 antibody conjugate (IMC) significantly inhibited the growth of BT-474 cells transplanted into athymic mice. The in vivo growth-inhibitory effect of IMC treatment was similar to that observed in animals receiving D5 continuously as a dietary supplement. The results showed that the targeted delivery of D5 by immunoconjugation to cell surface receptor antibodies may be of potential therapeutic value for the treatment of Her-2 positive breast cancer (Punj 2004).
7. Dr. Vijayakumar and his colleagues had a poster presentation accepted for the 2004 annual meeting of the American Radium Society. Their poster, "Clinical Trial Design in Chemoprevention Studies: Using a Vitamin D5 Analog Study as an Example," highlights the novelty of the trial design with D5, which combines phases I (dosage and toxicity) and II (efficacy). There are significant design issues in chemoprevention studies, especially post radiotherapy (RT). Important aspects to be considered are serum and tissue intermediate biomarkers. The PI has designed a randomized Phase I/II study with end-of-study tissue biopsy, enabling assessment of tolerance, compliance, Quality of Life and intermediate bio-/molecular markers (Vijayakumar 2004).

REPORTABLE OUTCOMES

1. Manuscripts and Abstracts: Seven manuscripts and abstracts have been written over the past year. Please see our list of "References" below, and descriptions of the research above (Appendix 4 has each article or abstract).
2. FDA approval is pending. When that occurs, we will have exclusive rights to use D5 for studies. FDA approval for D5 is being pursued by the principal investigator's colleagues at the University of Illinois at Chicago and is quite close to being done. Dr. Tapas Das Gupta reports that after completing pre-clinical toxicity studies in dogs and rats some time ago, he submitted an application to the FDA for approval of D5. Currently he continues to correspond with the FDA on the matter. During his second submission to the FDA, the FDA had a few questions on the stability of the formulation of D5. Dr. Das Gupta accordingly is conducting a few more studies and once the data from these are submitted he anticipates securing FDA approval for the D5 trial. FDA approval may occur as soon as April 2004. A letter from Dr. Das Gupta, confirming the availability of D5 for the study, is attached as part of the appendix (see Appendix 3).

CONCLUSIONS

We have submitted the protocol to our IRB, and they have granted provisional approval, pending minor revisions. After we make those revisions and official IRB approval is obtained, we plan to apply for DOD approval. FDA approval for D5 is also pending and we await that. We hope to start the clinical trial later this year.

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2. Graves J, Babbar D, Hussain EA, Kute TE, Das Gupta TK, and Mehta RR. Therapeutic potential of 1 α (OH)D5 in herceptin-resistant breast cancer (#1956). Proceedings of the American Association for Cancer Research 2003 Mar;44:446.
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7. Vijayakumar S, Mehta RG, Mehta RR, Hall W, Boerner P and Beckett L. Clinical trial design in chemoprevention studies: using a vitamin D5 analog study as an example (#145). 86th Annual Meeting of the American Radium Society 2004 May; Napa, Calif.

APPENDICES

1. Letter from Dr. Vijayakumar to CCSRC, dated January 13, 2004
2. UCD Cancer Center Scientific Review Committee (CCSRC) Letter of Approval
3. Letter from Dr. Das Gupta at UIC
4. Seven published articles and abstracts from 2003-04 (see "References" list above).
5. Protocol Sent to UC Davis IRB on February 19, 2004
6. UC Davis IRB Letter of Approval, Pending Minor Revisions

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January 13, 2004

Primo Lara, M.D.
Co-Chair
Cancer Center Scientific Review Committee
Cancer Center
UCDMC

RE: Study UCDC#141, "*A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study*"

Dear Dr. Lara:

Thank you very much for reviewing our protocol at the December 11, 2003 meeting of the Cancer Center Scientific Review Committee. The Committee members' suggestions were helpful to us.

The Committee approved of our protocol, with recommendations. Specifically, we were to create a Treatment Plan section (reflecting toxicology-related dose modifications) and a drug diary. Attached please find these two additional parts of the protocol, which will be included in the version that is going on to the IRB.

If you have any questions about this submission, please do not hesitate to contact me.

Sincerely,

A handwritten signature in black ink, appearing to read "Srinivasan Vijayakumar", is written over a horizontal line.

Srinivasan Vijayakumar, M.D.
Professor and Chair
Department of Radiation Oncology

Enclosures: 1. Treatment Plan for D5 Protocol
2. Pill Diary Form

SV:pb

Cancer Center Scientific Review Committee
APPROVAL FORM

12/11/04

SRC Meeting Date: _____

Principal Investigator: _____
Srinivisan Vijayakumar, M.D.Disease Site: _____
GenitourinaryDisease Sub site: _____
ProstateStudy Number: UCDCC#141 Sponsor: Department of DefenseProtocol Title: A Phase I/II Double-Blinded, Randomized Clinical Trial To Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1a-Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking StudyTotal Target Accrual: 40UCD Target Accrual: 40

Does protocol involve Lawrence Livermore National Laboratory (LLNL)?

☐ Yes – copy of form, SRC minutes and comments sent to LLNL IRB☒ No

Voting Outcome:

☒ Approved☐ Approved with recommendations (explain below)☐ Approved with mandatory revisions - resubmission to SRC required (explain below)☐ Disapproved (explain below)☐ Tabled (to be reconsidered at next SRC meeting)☐ Withdrawn

Explanation/Additional Comments:

Co-Chair Signature (Primo Lara, M.D.)_____
Date

NOTE: Original must be included in IRB submission package

UNIVERSITY OF ILLINOIS
AT CHICAGO

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Tapas K. Das Gupta, MD, PhD, DSc
Professor and Head

January 20, 2004

Srinivasan Vijayakumar, MD
Head, Department of Radiation Oncology
University of California-Davis
4501 X Street, Suite G-126
Sacramento, CA 95817

Dear Dr. Vijay:

I am writing this letter to confirm our willingness to provide you with the data generated from the Phase I clinical trial with 1α -Hydroxyvitamin D5 in breast cancer patients and its current status. As you know we have completed the preclinical toxicity studies in dogs and rats under GLP guidelines sometime back. Since then we have submitted our application to the FDA for the approval to initiate this trial. Currently we are in correspondence with the FDA. During our second submission, the FDA has again pointed out a couple of deficiencies in relation to the stability of the drug product. These studies are in progress and once these data are submitted we should be able to obtain approval for the trial and initiate the study. We anticipate an additional 2-3 months for this to be completed. These results once generated in patients will be useful for your studies and clinical trial for prostate cancer patients.

With best regards,

Sincerely,

Tapas K. Das Gupta

Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

TKD:nc

UIC

2-8-selective inhibitor, rottlerin, enough inhibition of $\Delta\Psi_m$ and cytochrome c release. ZVAD-FMK (200 μM) blocks cytochrome c release, but does not inhibit $\Delta\Psi_m$ and cytochrome c release. To achieve apoptotic activation, we also used a combined EGTA/BAPTA-AM. A treatment of intact cells results in these results suggest that AD-198 induces cytochrome c release through a mechanism involving mitochondrial Ca^{2+} influx. AD-198 is consistent with the induction of apoptosis. (Supported by The National Institutes of Health, National Cancer Institute, and the National Science Foundation.)

arsenic trioxide (As₂O₃) at low induces complete remission with a promyelocytic leukemia (APL). started at an early stage of differentiation of both cell types. We have cell viability of cultured NB cells, in a relapsed tumor with acquired O₃ also inhibited NB growth of passage was activated in the treated did not inhibit itself in the Cytoc suggesting transcriptional involve- and bax expression. The dig- bax was induced in a form of bax, localized in particulate fraction to the pro-apoptotic bax inhibitor caspase-9. In contrast to induction of bax in NB (carbo- the expression of bax in NB cells expressed. We conclude that a bax expression in neuroblastoma may be a marker for bax in- duction in NB.

[illegible]

tion when exposed to extremely low *in vitro* concentrations of CDDO, 8–32 nM. Unlike the NBT reactivity and the cytostatic/cytocidal activities of trans-retinoic acid which overlap in concentration range and which change over time, the NBT reactivity and cytostatic/cytocidal activities of CDDO occur over separate concentration ranges and do not change over time. Results of this study confirm and extend the initial observations reported by others and serve as a further basis for NCI to pursue more detailed pre-clinical efficacy and toxicology evaluations on these novel agents.

#5536 Effects of 1 α -hydroxyvitamin D5 on prostate cancer cells. Divya Babbar, Mona Gandhi, Rajendra G. Mehta, Srinivasan Vijayakumar, and Rajeshwari R. Mehta. *University of Illinois at Chicago, Chicago, IL.*

Epidemiological studies have suggested that vitamin D deficiency plays an important role in the etiology and prevention of prostate cancer. An active metabolite of vitamin D3, 1,25 (OH)2D3, has been shown to have potent growth-inhibitory and cell-differentiating actions in prostate cancer cells; however, the use of 1,25 (OH)2D3 as a therapeutic agent is limited due to its severe toxicity. We recently showed that a synthetic analog of vitamin D, 1 α (OH)D5, has cell-differentiating and antiproliferative actions in breast cancer cells, and detailed preclinical studies indicated that this analog is tolerated at a very high concentration without causing severe toxicity. In this study, we evaluated the effects of 1 α (OH)D5 (D5) on androgen-sensitive and VDR-positive LNCaP and androgen receptor-negative DU-145 prostate cancer cells. Immunocytochemical studies of LNCaP cells incubated for 4 days in the presence of 0.1-1 μ M D5 showed an increase in cell surface expression of cytokeratin 8 and cytokeratin 18 and intracellular expression of PSA and IGFBP-3 as compared to those cells incubated in the presence of vehicle only. Western blot analysis further confirmed that D5 treatment increased the levels of CK-8 (54 kD), CK-18 (45 kD), PSA (33 kD), and IGFBP-3 (43 kD) in only LNCaP cells. D5 treatment also showed up-regulation of VDR. Du-145 cells failed to show any effects on the above-referenced differentiation biomarkers for prostate cells. Only in LNCaP cells did we observe reduced percentage of cells staining for Ki-67 in D5-treated cells as compared to control vehicle-treated cells. Flow cytometric analysis suggested that, following D5 treatment, LNCaP cells accumulate in S-G2 phase and D5 also induces apoptosis. Microscopic studies of LNCaP cells suggest that, following D5 treatment, an increased percentage of large cells with multiple fragmented nuclei surrounded by thin cytoplasm is observed (a characteristic of cells undergoing senescence). We studied β -galactosidase activity as a marker of senescence using x-gal as a substrate. In cells treated with D5, we observed an increased percentage of cells staining for β -galactosidase activity as compared to those treated with vehicle only. Our results in the present study suggest that D5; a noncalcemic analog, induces differentiation in prostate cancer cells. It also has antiproliferative actions and induces senescence followed by apoptosis in prostate cancer cells. These results suggest that 1 α (OH)D5 may be a valuable therapeutic agent for prostate cancer treatment. (This work was supported in part by US Army Award DAMD-17-02-1-0070)

#5537 Conversion into osteoblasts of a human salivary cancer cell line secreting bone morphogenetic protein in response to 22-oxa 1 α , 25-dihydroxyvitamin D₃. Mitsunobu Sato, Daisuke Uchida, Yoshihisa Tateishi, Hiroki Iga, and Hideo Yoshida. *2nd Dept of Oral and Maxillofacial Surgery, Tokushima Univ Sch of Dentistry, Tokushima, Japan.*

Bone morphogenetic protein (BMP) signaling regulates differentiation of cells in both ectodermal and mesenchymal origins. We found that a neoplastic clonal cell line (HSG-AZA3) secreting BMP-2, which was prepared by 5-azacytidine treatment of neoplastic human salivary intercalated duct cell line HSG, can change into osteoblasts in response to 22-oxa-1 α , 25-dihydroxyvitamin D₃ (22-oxa-1 α , 25(OH)₂ D₃) in nude mouse, diffusion chamber or in vitro and that growth of the treated cells is significantly suppressed. Expression of osteopontin, osteonectin and core binding factor 1 (Cbfa1) was observed by RT-PCR or in situ hybridization in the treated HSG-AZA3 cells alone. In addition, it was found that the expression level of BMP2 mRNA in HSG-AZA3 cells was increased about twofold as compared with the HSG cells and that secretion of BMP2 protein was detected by ELISA in HSG-AZA3 cells, but not in HSG cells. Moreover, expression of transcriptionary intermediary factor (TIF2) which is selectively recruited to complex of vitamin D receptor with 22-oxa-1 α , 25(OH)₂ D₃ was detected in the nuclei of HSG-AZA3 cells. It was found by RT-PCR that the expression levels of BMP receptors (type IA, IB, II) and Smads (1,5,8) mRNA in HSG-AZA3 cells were similar to those in HSG cells. Furthermore, it was found that Smad 1 protein in HSG-AZA3 cells, but not in HSG cells was phosphorylated, indicating that the autocrine loop of BMP2 signaling in HSG-AZA3 cells might function. To clarify the function of BMP2-Smads signaling, constitutively active BMP type IA receptor (CA-ALK3) was overexpressed in both HSG-AZA3 and HSG cells and expression

of Cbfa1 mRNA, which is induced by BMP-2, was subsequently, similar level of Cbfa1 mRNA expression in AZA3 and HSG cells, indicating that the molecular signaling pathway well work in both cells. These results suggest that the osteoblasts of HSG-AZA3 cells after treatment might be due to BMP2 secretion as well as to TGF- β 1.

#5538 Cathepsin D Induction Plays a Negative Role in Tumor Cell Differentiation
Lijuan Xia, Gen Sheng Wu, Samuel Waxman, & Zhenming Chen
School of Medicine, New York, NY and Wayne State University, Detroit, MI

Cathepsin D is an acidic lysosomal protease in hemotopoietic cells and is not expressed in acute HL-60 cells. All-trans retinoic acid (ATRA) in a dose and time dependent manner in both NB4 and with differentiation induction. ATRA does not in R4, MR-2 and HL-60/Res cells which are resistant. Previously we reported that As2O3 overcomes MR-2 not in HL-60/Res cells. Correlated with cathepsin D expression is induced in R4 and As2O3 in combination with ATRA. By using receptors, we find that ATRA, Am80, Am580 acid (ligand of both RAR and RXR), but not L cathepsin D expression and differentiation. ATL D promoter in a transient transfection assay. This is not a primary ATRA response gene and its induction differentiation initiation. Based on these data it contributes to ATRA-mediated differentiation differentiation is enhanced by 50% by cotreatment cathepsin D inhibitor). This suggests that it plays a negative role in ATRA-induced cell differentiation known pro-apoptotic effect. Cathepsin D inhibition with ATRA for more effective differentiation leukemia.

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#5539 Relationship between COX-2 and
Atsushi Mizokami, Hirotaka Okahigashi, Ki
Youti Iwasa. *Department of Urology, Kanazawa*

Most of prostate cancers are generally resistant to anti-cancer drugs. In order to know drug resistance in prostate cancer, we evaluated LNCaP cells which are overexpressing COX-2. We employed several anti-cancer drugs, CDDP, VP-16, and paclitaxel, and COX-2 and LNCaP-neo cells which do not express COX-2 cells were more resistant to CDDP. There were no differences in IC50 of other agents. We investigated the expression level of several genes. COX-2 mRNA level was significantly increased in LNCaP-COX-2 cells with CDDP and NS398 increased the sensitivity to CDDP. CDDP inhibitor may be effective in the case of treatment of prostate cancer.

#5540 Overexpression of the human sp
tidrug resistance to anti-cancer agents. W:
Susan Pratt, Nathalie Godinot, Mark Williar.
Shengfang Jin, Andrew W. Shyjan, Mark R
Dantzig. Lilly Research Laboratories, Indiana
ticals, Inc., Cambridge, MA.

Alternate splicing is a powerful mechanism for generating protein diversity. The 45 kDa human protein known to regulate alternate splicing through activation of alternate 3' splice sites, SPF45 was overexpressed in a majority of colorectal cancers, suggesting altered expression might be important in colorectal cancer.

We have shown previously that 1 α , 25-dihydroxy-21-(3-hydroxy-3-methyl-butyl)vitamin D₃ (Gemini) compounds, which have two side chains attached to carbon-20, had increased anti-tumor activities against breast, prostate and leukemic cell lines in comparison to 1,25-dihydroxy-vitamin D₃. Gemini compounds with additional modifications were synthesized and their anticancer activities were evaluated. Most effective in this series was 1,25-dihydroxy-20S-21-(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluoro-vitamin D₃ (Gemini-23-yne-26,27-hexafluoro-D₃). This analog was approximately 10-fold more potent than previously characterized Gemini compounds in inhibiting 50% of the clonal growth (ED50) of HL-60 (myeloid leukemia), MCF-7 (breast cancer) and LNCap (prostate cancer) cell lines at doses of 1.6x10⁻¹⁰M, 1.7x10⁻¹⁰M, 7.8x10⁻¹⁰M, respectively. Culture of HL-60 cells for 4 days with Gemini-23-yne-26,27-hexafluoro-D₃ (10⁻⁸M) caused their cell cycle arrest, and stimulated expression of CD14 (40%), a marker of macrophage-like differentiation. Also, in MCF-7 cells Gemini-23-yne-26,27-hexafluoro-D₃ stimulated protein expression of PTEN in a dose- and time-dependent manner. PTEN can act as a tumor suppressor by dephosphorylating the phosphatidylinositol 3' kinase (PI3K), resulting in reduced phosphorylation and activation of the oncogenic kinase, Akt. Gemini-23-yne-26,27-hexafluoro-D₃ suppressed expression of phosphorylated Akt, resulting in dephosphorylation of the Akt target proteins Bad, Forkhead transcription factor and mammalian target of rapamycin (mTOR). Downstream effectors of mTOR were also inhibited by the analog as demonstrated by decreased phosphorylation of both the mitogenic p70 S6 kinase, and the translation inhibitor, 4E-BP1. The effects of Gemini-23-yne-26,27-hexafluoro-D₃ on the Akt pathway were not seen when MCF-7 cells were co-cultured with the PI3K inhibitor, LY294002. These results suggest some of the antiproliferative properties of vitamin D₃ analogs in MCF-7 cells are mediated via modulation of the PI3K/Akt signaling pathway.

#1956 Therapeutic potential of 1 α (OH)D5 in herceptin-resistant breast cancer. Jewell Graves, Divya Babbar, Erum A. Hussain, Timothy E. Kute, Tapas K. Das Gupta, and Rajeshwari R. Mehta. *University of Illinois at Chicago Medical Center, Chicago, IL and Wake Forest University School of Medicine, Winston-Salem, NC.*

Human breast cancers showing overexpression of Her-2 protein are highly aggressive. Herceptin, a humanized Her-2 antibody, has recently been used to treat metastatic breast cancer. Even though Her-2+ patients do show response, many patients show disease progression after herceptin treatment. We studied the effect of a new nontoxic vitamin D analog, 1 α (OH)D5, on a herceptin-resistant human breast carcinoma cell line. Herceptin resistant breast cancer cell line (BTR) was generated by incubating BT-474 cells continuously for > 6 months in the presence of herceptin (10 μ g/ml). In vitro, authentic BT-474 cells showed a growth-inhibitory effect (40%) when incubated for 7 days with Herceptin (10 μ g/ml) as compared to cells incubated in the presence of isotype-matched IgG control. Herceptin had no effect on growth of BTR cells. Comparative analysis of BT-474 and BTR cells indicated that BTR cells express higher levels of Her-2 receptor P-1-3K proteins. The effect of dietary supplement of (D5) was evaluated on BTR cells transplanted into 3-4 week old female athymic mice. D5 was supplemented in the diet at 12.5 μ g/kg diet. Animals received control diet or received D5-supplemented diet. In vivo, animals receiving D5-supplemented diet had significantly (p<0.05) smaller tumors after 14 days of diet as compared to animals in control group receiving regular diet. At termination, tumors were excised and processed for immunohistochemical/biochemical studies for expression of various proteins. Xenograft tumors originated in animals receiving D5 diet had significantly less % of Ki-67 positive staining as compared to those in control group. Similarly % of cells expressing nuclear immunostaining for cyclin D1 and cyclin A was less but p27 was higher in xenograft originated in D5 treated animals. D5 had no effect on Her-2, PI-3-kinase AKT protein levels. Blood calcium levels were similar in both control and D5-treated animals. These results suggest that this noncalcemic vitamin D analog mediates its action by regulating the cell cycle-associated proteins in herceptin-resistant breast cancer cells and may be considered for its therapeutic value for treatment of herceptin-resistant breast cancer. (Supported in part from funding provided by the Penny Severns Breast Cancer and Cervical Research Funds, Illinois Department of Public Health, and Department of Defense Breast Cancer Research Program (DAMD17-9-1-7263 and DAMD17-99-1-9223). We would like to acknowledge Genentech for generous supply of herceptin.)

#1957 Dynamic profiling of sex steroid and EGF-signaling pathway in rat uterus by genistein. Michelle S. Cotroneo, Wayne Fritz, and Coral A. Lamartiniere. *University of Alabama at Birmingham, Birmingham, AL.*

The Asian diet, high in soy products, is associated with reduced incidence of hormone-dependent cancers, including endometrial cancer. The primary isoflavone component of soy is genistein, for which we have investigated the time course expression of regulating sex steroid receptor expression and the epidermal growth

factor (EGF) signaling pathway in the rat uterus. Female Sprague Dawley (SD) rats (30 days old) were killed 0, 2, 4, 8, 16, 24, and 48 h after 1 injection of genistein/g BW, 500 ng estradiol benzoate (EB)/g BW or the vehicle, dimethyl sulfoxide (DMSO). Estrogen receptor (ER)- α , decreased after treatment with genistein or EB within 2 h, returning to basal levels within 24 and 48 h. Subsequently, progesterone receptor (PR) was up-regulated at 24 and 48 h, consistent with the idea of PR being a "late" gene product, indicative of differentiation. EGF-receptor (EGFR) expression peaked 16 h after genistein treatment, inversely correlating with extracellular-regulated kinase (ERK) phosphorylation. The up-regulation of EGFR in the uterus is contrary to in vitro of genistein being an inhibitor of protein tyrosine kinases, but consistent with previous demonstration that genistein initially up-regulates EGFR expression in mammary. The percentage of phosphorylated EGFR, relative to total EGFR, and phosphorylation of the downstream kinases Raf-1 and MAP kinase/ERKs (MEKs) 1 and 2 were reduced, corresponding with decreased phosphorylation in the stroma, suggesting signal attenuation, and a delay in differentiation. This demonstrates that protein phosphorylation is an important component of receptor protein levels. At 16 h, we observed increased weight, epithelial cell height, and cell proliferation in the stroma and endometrium. These effects were inhibited by pretreatment with the antiestrogen, ICI 164,384, suggesting a requirement for ER. Pharmacokinetic analysis showed that the concentrations of genistein and EB at 5.41 and 0.92 h, respectively. These results suggest similar mechanisms of action for genistein and EB in induction of uterine differentiation, but with 1/1000 potency. (Supported by NIH RO1 CA61724, 00-1-0118, NIH 5 R25 CA4788 and DOD 17-00-1-0119.)

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Mechanisms and Modulation of Drug Resistance

#1958 Overexpression of E2F-1 sensitizes cells to mitotic phase drugs. Angelo Joseph Russo, Pellegrino Magro, Debabrata Banerjee, Zhen Hu, and Joseph R. Bertino. *Cancer Institute Of New Jersey (UMNJ), Brunswick, NJ and Memorial Sloan-Kettering Cancer Center, New York, NY.*

Several reports have demonstrated the critical role that the transcription factor E2F-1 plays in coordinating transcription of specific genes essential for cell division. In the cell cycle, hypophosphorylated pRB binds E2F-1, inhibiting its transcriptional activity. In early G1, pRB becomes phosphorylated by cyclin-dependent kinases, disrupting the pRB binding to E2F-1, allowing E2F-1 to act as a transcription factor involved in proliferation. The role of E2F-1 in affecting cytotoxicity of chemotherapeutic drugs has been examined with varying results. In the present study, we investigate the role of E2F-1 in cytotoxicity of various chemotherapeutic agents. Upon overexpression of E2F-1, doxorubicin, cisplatin, and etoposide all showed increased play any differences in cytotoxicity profiles using the SRB method. In contrast, overexpression of E2F-1 caused a dramatic sensitivity to mitotic phase drugs, namely vinblastine and paclitaxel. Recent reports have implicated the E2Fs in the G2/M checkpoint of the cell cycle. Therefore, we investigated the effect of E2F-1 overexpression on important G2/M regulatory genes. Overexpression of cyclin B1 and cdc2 in order to obtain an understanding of the sensitivity of E2F-1 overexpression, cyclin B1 protein levels increased dramatically. After 16 hours of induction with a significant increase in mRNA levels and cyclin-dependent cdc2 kinase activity. Furthermore, in experiments in which E2F-1 was overexpressed in the presence of 100nM paclitaxel an increase in cyclin B1 protein levels as well with an associated increase in mpm-2 protein expression were observed for cells in mitosis. Based on preliminary results demonstrating that luciferase activity upon overexpression of E2F-1, current studies are underway to understand the mechanism of regulation of the cyclin B1 promoter.

#1959 Imatinib mesylate (STI571, Gleevec®) is a potent inhibitor of ABCG2 and reverses topotecan resistance in vitro. Peter J. Houghton, Germain, John D. Schuetz, Clinton F. Stewart, Elisabeth Buchdunger, Traxler. *St. Jude Children's Research Hospital, Memphis, TN and Novartis AG, Basel, Switzerland.*

Although selective tyrosine kinase inhibitors (TKIs) have demonstrated antitumor activity alone, development of novel therapies with the necessary combination with conventional cytotoxic agents. The assumption is that in combination any interaction between the TKI and the drug (i.e. synergy) will be mediated through inhibition of the TKI kinase. However, to build rational combinations with TKIs this assumption must be tested. Previously we reported that a 4-anilinoquinazoline inhibits

Efficacy and Mechanism of Action of 1α -hydroxy-24-ethyl-Cholecalciferol (1α [OH]D5) in Breast Cancer Prevention and Therapy

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Abstract

It is now well established that the active metabolite of vitamin D₃, $1\alpha,25(\text{OH})_2\text{D}_3$, regulates cell growth and differentiation in various in vitro cancer models. However, its clinical use is precluded due to its hypercalcemic activity in vivo. Hence, several less calcemic vitamin D analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. A novel analog of vitamin D₃, 1α -hydroxy-24-ethyl-cholecalciferol (1α [OH]D5), has currently been under investigation in our laboratory for its application in breast cancer prevention and therapy. 1α (OH)D5 had been shown to inhibit development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect was more significant if 1α (OH)D5 was present during the promotional phase of the lesion development. The growth inhibitory effect of 1α (OH)D5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α (OH)D5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor-negative (VDR-) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α (OH)D5. This suggests the requirement of VDR in 1α (OH)D5-mediated growth effects. Interestingly, breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER- cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α (OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α (OH)D5 treatment. This implies a differential effect of 1α (OH)D5 on ER+ vs. ER- cells. Additionally, comparison between the effects of 1α (OH)D5 on normal vs. transformed cells indicated that 1α (OH)D5 does not suppress cell prolifera-

tion of normal epithelial cells but selectively targets growth of transformed cells. We extended our experiments to determine *in vivo* effects of $1\alpha(\text{OH})\text{D}_5$ using an MNU-induced mammary carcinogenesis model in female Sprague-Dawley rats. Results showed that $1\alpha(\text{OH})\text{D}_5$ (25–50 $\mu\text{g}/\text{kg}$ diet) decreased the incidence and multiplicity of mammary tumors in these rats. In addition, it increased the latency period of early precancerous lesions. Similar studies, with DMBA as a carcinogen in younger rats, showed that $1\alpha(\text{OH})\text{D}_5$ supplementation was effective in reducing onset of carcinogenesis in rats and the effect was largely reflected during the promotional phase of carcinogenesis. Recently, a preclinical toxicity profile for $1\alpha(\text{OH})\text{D}_5$ was completed in rats and dogs that provides estimation of the maximum tolerated dose in mammals. Based on our findings, we will shortly be initiating a $1\alpha(\text{OH})\text{D}_5$ phase I clinical trial for breast cancer patients.

Introduction

Breast cancer is generally characterized by transformation of normal to atypical hyperplastic epithelium, with subsequent risk of progression to intraductal carcinoma and in some cases invasion into stroma (Mallon et al. 2000). Breast cancer is the second leading cause of cancer-related deaths among women in the US, with about 180,000 new cases and 46,000 deaths annually (Edwards et al. 2002). Although the overall incidence of breast cancer has not been reduced in the last decade, the breast cancer-related mortality has been decreasing with approximately 3.4% annual decrease from 1995 through 1998 in the US (Howe et al. 2001; Peto et al. 2000). This decrease in mortality is probably a result of availability of greater screening efficiency and better chemopreventive and therapeutic strategies. Despite increased survival rates, breast cancer results in considerable morbidity and patient care costs. Chemoprevention is an important aspect of curbing the progression or recurrence of the disease. The chemopreventive agents usually include natural or synthetic compounds that can either prevent transformation or inhibit proliferation of transformed cells by inducing apoptosis, growth arrest or differentiation of initiated and transformed cells (Rosenbaum-Smith and Osborne 2000). Several classes of compounds have been under investigation in this regard. These include selective estrogen receptor modulators, retinoids, diltanoids (vitamin D derivatives), phytoestrogens, flavonoids, and aromatase inhibitors, among others (Kelloff et al. 1996).

On a global basis, breast cancer incidence is fivefold higher among middle-aged women in the Western countries than in women from Asian countries. Various diet and lifestyle as well as genetic factors have been implicated in the high occurrence of breast cancer in the Western world. Some epidemiological studies have shown association of lower sunlight exposure to higher breast, colon, and prostate cancer mortality rates in the US and other Western countries (Freedman et al. 2002; Polek and Weigel 2002; Garland et al. 1990; Gorham et al. 1990). This is consistent with reports of an association of breast

cancer mortality with lower serum vitamin D₃ levels (John et al. 1999; Christakos 1994). Lower serum vitamin D₃ levels could be due to lower sunlight exposure as well as lower dietary intake.

The biologically active metabolite of vitamin D, 1 α ,25(OH)₂D₃ or calcitriol, is a steroid hormone that was identified in the early 1920s as an antirachitic substance (Carpenter and Zhao 1999). Later it was established that vitamin D₃ is synthesized in the skin from 7-dehydrocholesterol by the action of ultraviolet radiation. Vitamin D₃ is activated subsequently in liver and kidney by the hydroxylation reactions at C25 and 1 α positions to yield 1 α ,25(OH)₂D₃. Calcitriol has been known to exert calcitrophic effects, mainly through increasing calcium uptake in the intestine for regulation of bone health. Aside from its role in calcium homeostasis, vitamin D₃ is involved in regulation of various cellular processes. Vitamin D₃ binds to nuclear vitamin D receptor (VDR) and undergoes conformational changes, which allow VDR to function as a transcription factor (Jones et al. 1998; Haussler 1986). Earlier, VDR was found to be present in abundance in intestine, bone, liver, and kidney cells. Aside from the classic target organs, VDR has now been isolated from a variety of tissues, including normal mammary epithelium as well as breast tumors (Friedrich et al. 1998; Buras et al. 1994; Eisman et al. 1980).

In order for VDR to function, it needs to interact with vitamin D response elements (VDRE) and bind to DNA to initiate or repress transcription (Pike 1991). VDR must form a dimer to stabilize VDRE transactivation (Jones et al. 1998). Most common partners for VDR heterodimerization are nuclear accessory factor (NAF) and retinoid X receptor (RXR) (Rachez and Freedman 2000). VDR transactivation of VDRE results in regulation of a wide variety of genes, some of which are involved in cell growth and proliferation. Vitamin D₃ also exerts some nongenomic rapid responses possibly through a putative membrane receptor (Falkenstein et al. 2000).

The presence of VDR in the normal mammary epithelial cells suggests a role of calcitriol in the regulation of mammary gland function. The levels of VDR in mammary tissue increase during pregnancy and lactation and decrease as the glands regress back to normal size (Zinser et al. 2002; Narvaez et al. 2001). VDR knockout mice have been shown to have larger mammary glands than normal mice; it has also been shown that the glands would not regress back to prepregnancy size at the termination of lactation (Zinser et al. 2002). This suggests that vitamin D mediated signaling may be very important for maintaining the normal cycling of the mammary gland. Various case studies indicate that a high percentage (60%–80%) of breast cancer epithelia contain VDR (Friedrich et al. 1998) and that there is a positive correlation between VDR polymorphisms and increased risk of breast cancer (Bretherton-Watt et al. 2001; Lundin et al. 1999). These reports further signify vitamin D₃-mediated signaling to be of importance in regulating healthy mammary gland. In cell culture models, vitamin D₃ has been demonstrated as an inducer of growth arrest and differentiation in various cancer cell lines, including breast cancer cells (Hisatake et al. 2001; Welsh et al. 1998; James et al. 1997). Taken together, these results warrant potential use of vitamin D₃ in cancer preven-

tion and therapy. However, due to its hypercalcemic activity, vitamin D₃ can not be administered at doses that would be effective for chemoprevention or therapy. Adverse effects of vitamin D₃ at cancer-preventive doses are hypercalcemia, soft tissue calcification, weight loss, and possibly death (Roder and Stair 1999; Vieth, 1999).

Since the early 1980s, there has been a search for a vitamin D₃ analog that would selectively modulate VDR to produce growth-regulating effects without interfering with the calcium metabolism. Several analogs have been synthesized and tested for this purpose; but only a few have shown promising results in cell culture and animal models. Vitamin D₃ analogs currently being evaluated for breast cancer prevention include seocalcitol (EB-1089), calcipotriol (KH-1060), Maxacalcitol (OCT), RO-24-5531, and 1 α (OH)D5 (Mehta and Mehta 2002; Guyton et al. 2001). In this review, we summarize the results from experiments conducted in our laboratory that elucidate the potential role of 1 α -hydroxy-24-ethyl-cholecalciferol (1 α [OH]D5) in breast cancer prevention or therapy.

Synthesis and Characterization of Vitamin D Analog, 1 α (OH)D5

As mentioned earlier, vitamin D₃ can be obtained from food as well as synthesized in the skin through the action of sunlight. Vitamin D₃ belongs to the family of 9,10-secosteroids which differ only in side-chain structure (Napoli et al. 1979). Other forms of D-compounds include D2, D4, D5, and D6. In the late 1970s, major interest in the synthesis of these compounds was to evaluate them for use in management of renal osteodystrophy and osteoporosis. In this regard the calcemic activity of D series of compounds was compared and D5 was found to be the least calcemic of all (Napoli et al. 1979), a property that would later prove useful in its possible application for cancer prevention. The D5 form is also known as irradiated 7-dehydrositosterol. The hydroxylated form of D5 (1 α [OH]D5) was synthesized as described previously (Mehta et al. 1997a).

Briefly, β -sitosterol acetate was converted to 7-dehydro- β -sitosterol acetate by allelic bromination and dehydrobromination. Lithium aluminum hydride and tetrahydrofuran were used to reduce 7-dehydro- β -sitosterol to 7-dehydro-3 β -sitosterol. The reaction mix was sequentially subjected to photolysis and thermolysis to yield 24-ethyl-cholecalciferol (D5). D5 was hydroxylated by Paaren-DeLuca hydroxylation sequence to produce 1 α (OH)D5. The product was crystallized and characterized by ¹H nuclear magnetic resonance at 400 Hz and mass spectroscopy. The purity was assessed by high-pressure liquid chromatography. The following properties were observed: melting point, 150–152°C; UV λ -max, 265 nm; molar extinction coefficient (ϵ), 18000; molecular weight, 428.7. The major structural differences between biologically active vitamin D₃ and 1 α (OH)D5 are the lack of hydroxylation at the C-25 position and the presence of an ethyl group at the C-24 position in the 1 α (OH)D5 molecule (Fig. 1).

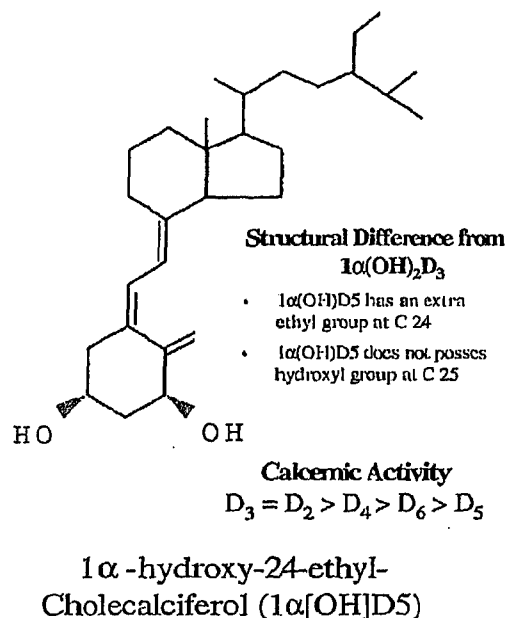


Fig. 1 Structure of 1 α (OH)D5 and its Ca⁺⁺ mobilizing activity in mammals in relation to other primary vitamin D series compounds

Calcemic Activity of 1 α (OH)D5

Earlier studies in DeLuca's lab had shown that among the known vitamin D series of compounds (vitamin D $_2$ -D $_6$), D5 is the least calcemic of all (Napoli et al. 1979). D5 was found to be 80-fold less active than vitamin D $_3$ in the intestine and about 100- to 200-fold less active in bone in mobilizing the Ca⁺⁺ stores (Napoli et al. 1979). The calcemic activity of the hydroxylated form was not known. Therefore, we measured calcemic activity as well as body weight change in animal models to determine the maximum tolerable dose and toxicity of 1 α (OH)D5. In the first experiment, 3-week-old Sprague-Dawley male rats were fed a vitamin D $_3$ -free diet containing 0.47 g calcium and 0.3 g phosphorus/100 g diet (Mehta et al. 1997a). These rats were kept under yellow light to create a vitamin D $_3$ -deficiency state. After the rats were fed a vitamin D $_3$ -deficient diet for 3 weeks, their plasma calcium levels were measured and rats with calcium levels under 6.0 mg/dl were considered vitamin D $_3$ deficient. Vitamin D $_3$ -deficient rats were administered 1 α (OH)D5 intragastrically for 14 days and the plasma calcium levels were measured. The control group showed a plasma calcium concentration of 5.4 \pm 0.3 mg/dl, while the rats receiving 1 α (OH)D5 at a dose of 0.042 μ g/kg per day had plasma calcium concentration of 6.0 \pm 0.63 mg/dl, which was not significantly different from the control rats (Mehta et al. 1997a). On the other hand, vitamin D $_3$ increased

Table 1 Calcemic activity of $1\alpha(\text{OH})\text{D}_5$ in Sprague-Dawley rats

Treatment	Sample size	Dose	Plasma Ca^{++} (mg/dl)
Vitamin D-deficient male rats		($\mu\text{g/kg}$ body weight)	
Control	8	0.0	5.4 ± 0.28
$1\alpha(\text{OH})\text{D}_5$	8	0.042	6.0 ± 0.63
$1\alpha(\text{OH})_2\text{D}_3$	8	0.042	$8.1 \pm 1.2^*$
Vitamin D-sufficient female rats		($\mu\text{g/kg}$ diet)	
Control	15	0.0	7.0 ± 1.19
$1\alpha(\text{OH})\text{D}_5$	15	25.0	7.4 ± 1.10
$1\alpha,25(\text{OH})_2\text{D}_3$	15	12.8	$8.5 \pm 1.17^*$

* Significantly different from control ($p < 0.05$)

plasma calcium concentration 50% over that of the control group (Table 1). During these experiments, the $1\alpha(\text{OH})\text{D}_5$ group did not differ in total body weight from control group. No other signs of toxicity were observed in $1\alpha(\text{OH})\text{D}_5$ -fed rats compared to controls.

In a separate experiment, female Sprague-Dawley rats were fed a diet supplemented with $1\alpha(\text{OH})\text{D}_5$ to determine its calcemic activity in vitamin D_3 -sufficient rats. Food was provided ad libitum. There was no body weight change at $50 \mu\text{g } 1\alpha(\text{OH})\text{D}_5/\text{kg}$ diet in vitamin D_3 -sufficient rats, while a dose of $12.8 \mu\text{g } 1\alpha,25(\text{OH})_2\text{D}_3/\text{kg}$ diet was sufficient to bring about significant weight loss in the animals (Table 1). Maximum tolerated dose was determined to be $50 \mu\text{g/kg}$ diet, based on the weight and calcemic activity of $1\alpha(\text{OH})\text{D}_5$ in these rats (Mehta et al. 2000a). In addition to these experiments, we also conducted toxicity studies under the GLP using rats and dogs. For rats, the dose at which signs of toxicity first appeared was $10 \mu\text{g/kg}$ body weight (equivalent to $100 \mu\text{g } 1\alpha(\text{OH})\text{D}_5/\text{kg}$ diet for a 150-g rat), which is twice the amount needed to bring about effective chemoprevention. However, the dogs had much lower tolerance for $1\alpha(\text{OH})\text{D}_5$ compared to rats. Based on these results, we are now conducting further studies to determine the appropriate and safe dose of $1\alpha(\text{OH})\text{D}_5$ for use in clinical settings.

Since vitamin D_3 exerts most of its effects through binding to VDR, we evaluated the ability of $1\alpha(\text{OH})\text{D}_5$ to bind to VDR. The binding affinity of $1\alpha(\text{OH})\text{D}_5$ to VDR was determined using competitive binding assays (unpublished data). Results showed that the binding affinity of $1\alpha(\text{OH})\text{D}_5$, in competition with radioactive $1\alpha,25(\text{OH})_2\text{D}_3$ to purified VDR ligand-binding domain is 1000-fold less than $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig. 2). The IC_{50} for $1\alpha(\text{OH})\text{D}_5$ was 100 pM , while for $1\alpha,25(\text{OH})_2\text{D}_3$, it was 0.08 pM . The lower binding affinity may explain the decreased calcemic activity of $1\alpha(\text{OH})\text{D}_5$. However, due to its lower calcemic activity, $1\alpha(\text{OH})\text{D}_5$ can be administered at much higher doses than $1\alpha,25(\text{OH})_2\text{D}_3$. This quality can allow use of $1\alpha(\text{OH})\text{D}_5$ for prevention in the general population as well as high-risk groups. It is also important to note that the in vivo VDR affinity to its ligand is tissue specific (Napoli et al. 1979), which could not be manifested in our experiments that were conducted using

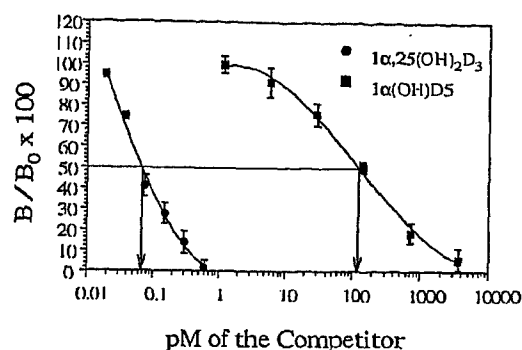


Fig. 2 Binding affinity of 1 α (OH)D₅ to VDR in comparison with 1 α ,25(OH)₂D₃

purified VDR. We have not yet critically evaluated metabolism and pharmacokinetics of 1 α (OH)D₅ in target organs.

Anticarcinogenic Effects of 1 α (OH)D₅ in In Vitro Models

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (MMOC). The mammary glands from balb/c mice are harvested and cultured in presence of appropriate hormones (Mehta et al. 1997b). These glands are subjected to short stimulation with a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA), which results in formation of precancerous preneoplastic lesions. When implanted in syngeneic hosts, the epithelial cells from these lesions give rise to adenocarcinomas. Effective chemopreventive agents would inhibit the development of these preneoplastic lesions. The chemopreventive activity of a compound in MMOC correlates very well with the activity in in vivo carcinogenesis models (Mehta et al. 1997b). Using a DMBA-induced MMOC model, Mehta et al. (1997a) showed that 1 α (OH)D₅ possesses chemopreventive activity. Fifteen mammary glands (per group) from balb/c mice were incubated with appropriate hormones and were exposed to the carcinogen DMBA (2 μ g/ml of culture media) on day 3 of a 24-day culture. The group of glands incubated with 1 α (OH)D₅ showed significant reduction of lesion formation compared to the control group (Fig. 3). Percent inhibition of lesion formation in each treatment group was calculated by comparing the incidences of lesions between the control and the treated group. A dose-response curve showed that 100% inhibition was achieved at 10 μ M 1 α (OH)D₅ concentration, but the optimal dose seems to be 1 μ M, as it shows significant (75%) inhibition without any signs of cytotoxicity. Vitamin D₃, on the other hand, caused dilation of ducts and disintegration of alveolar structures as signs of toxicity at 1 μ M concentration. Based on the MMOC model, 1 μ M 1 α (OH)D₅ seems to be equivalent in potency to 0.1 μ M 1 α ,25(OH)₂D₃.

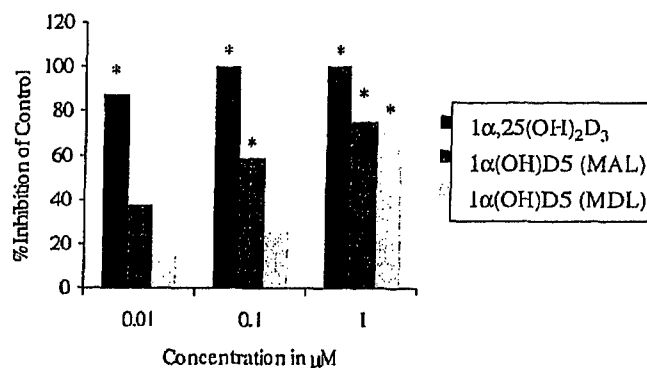


Fig. 3 Chemopreventive efficacy of $1\alpha(\text{OH})\text{D}_5$ in inhibiting mammary alveolar (MAL) and ductal (MDL) lesions in mouse mammary gland organ culture in comparison to $1\alpha,25(\text{OH})_2\text{D}_3$

In order to establish the stage specificity for the effectiveness of $1\alpha(\text{OH})\text{D}_5$ in a DMBA-induced MMOC model, $1\alpha(\text{OH})\text{D}_5$ was added either prior to or subsequent to carcinogen treatment. The initiation-only group received $1\alpha(\text{OH})\text{D}_5$ for the first 4 days of culture, whereas the promotion-only group received the treatment after withdrawal of carcinogen (days 4–10). Results indicated that $1\alpha(\text{OH})\text{D}_5$ is more effective when present during the promotional stages of lesion formation (Mehta et al. 2000a). In addition to inhibition of lesion formation, $1\alpha(\text{OH})\text{D}_5$ was effective in inducing VDR and $\text{TGF}\beta 1$ expression in mammary epithelial cells of MMOC. VDR and $\text{TGF}\beta 1$ expression was measured using immunohistochemistry. Briefly, paraffin-embedded sections were rehydrated, fixed, permeabilized, and incubated with primary antibody. The primary antibody binding was detected using biotinylated link and peroxidase-conjugated streptavidin, which was then visualized by 3-amino-9-ethyl-carbazole as chromogen. The mammary epithelial cells, which stained negative for VDR, failed to show $\text{TGF}\beta 1$ induction upon $1\alpha(\text{OH})\text{D}_5$ treatment. This implies the involvement of VDR in $1\alpha(\text{OH})\text{D}_5$ -mediated effects. The extent of induction of VDR and $\text{TGF}\beta 1$ upon treatment with $1.0 \mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ was similar to that observed with $0.1 \mu\text{M}$ vitamin D_3 (Mehta et al. 1997a). Despite the 1000-fold lower affinity of $1\alpha(\text{OH})\text{D}_5$ for VDR in comparison to $1\alpha,25(\text{OH})_2\text{D}_3$, its chemopreventive activity is equivalent to $1\alpha,25(\text{OH})_2\text{D}_3$ at only a 100-fold higher concentration. Therefore, it seems likely that the antiproliferative effects of $1\alpha(\text{OH})\text{D}_5$ may not be dependent solely upon its in vitro interactions with VDR.

Since the MMOC experiments involved the whole organ, the actions of $1\alpha(\text{OH})\text{D}_5$ on breast epithelia itself were not clearly established. Hence, we tested the growth effects of $1\alpha(\text{OH})\text{D}_5$ on various breast cancer cell lines of epithelial origin. All the cell lines tested were purchased from ATCC (Manassas, VA, USA), except UIISO-BCA-4 cells. This cell line was established in our laboratory from metastatic pleural fluid obtained from a 56-year-old woman with a confirmed diagnosis of breast carcinoma (Mehta et al. 1992). The

Table 2 Growth response of various breast cancer cell lines to 1 α (OH)D5 treatment

Cell lines	VDR status	ER status	PR status	Inhibition (%) ^a	Net effect of 1 α (OH)D5
BT-474	+	+	+	50	Cell cycle arrest, apoptosis
MCF-7	+	+	+	45	Cell cycle arrest, apoptosis
ZR-75-1	+	+	+	30	Growth inhibition
T-47D	+	+	+	30	Growth inhibition
UISO-BCA-4	+	-	-	40	Growth inhibition, differentiation
MDA-MB-231	-/+ (?)	-	-	None	None
MDA-MB-435	-	-	-	None	None

^a Percent growth inhibition at 1 μ M 1 α (OH)D5 for 72 h, adjusted for control

growth effects of 1 α (OH)D5 were assessed on BT-474, MCF-7, ZR-75-1, T-47D, UISO-BCA-4, MDA-MB-231 and MDA-MB-435 cell lines using multiple measures: cell counter, MTT absorbance assay (Twentymen and Luscombe 1987), and cell cycle analysis with propidium iodide staining and flow cytometry (Vindelov et al. 1983). The overall effects of 1 α (OH)D5 on the growth of different cell lines are summarized in Table 2. All the cell lines that were positive for VDR showed significant growth inhibition ($p < 0.05$) after 72 h of incubation with 1 α (OH)D5. BT-474, and MCF-7 (VDR+ ER+ PR+) cells showed the greatest growth inhibition and G-1 cell cycle arrest upon 1 α (OH)D5 treatment. Similarly, UISO-BCA-4 (VDR+ ER- PR-) cells exhibited growth inhibition in response to 1 α (OH)D5 treatment. On the other hand, VDR- MDA-MB-231 and MDA-MB-435 cells did not show any growth inhibition at 1 μ M 1 α (OH)D5 treatment (Mehta et al. 2002). The dose-response curve for 1 α (OH)D5 effect in BT-474 cells was similar to that observed in the MMOC experiments.

Chemopreventive Efficacy of 1 α (OH)D5 in In Vivo Carcinogenesis Models

Once we established the in vitro efficacy of 1 α (OH)D5, the effects of 1 α (OH)D5 were evaluated in experimental mammary carcinogenesis models. We used mammary-specific carcinogen *N*-methyl-*N*-nitrosourea (MNU) in rats to induce tumors and evaluated the efficacy of 1 α (OH)D5 to prevent or delay the incidence of mammary cancers in these rats (Mehta et al. 2000a). Fifteen Sprague-Dawley female virgin rats per group (9 weeks old) were fed 1 α (OH)D5-supplemented diet (25 or 50 μ g/kg) for 2 weeks before the carcinogen treatment. The carcinogen MNU was given as a single intravenous injection of 50 mg acidified MNU/kg body weight at 80 days of age. The rats continued to receive the 1 α (OH)D5-supplemented diet until they were killed at 190 days of age. The tumor incidence in control rats was 80%, which, compared to controls, decreased in 25- and 50- μ g/kg diet group by 33% and 42%, respectively (Table 3). The tumor incidence in the low-dose group was not sig-

Table 3 Efficacy of 1 α (OH)D5 in preventing carcinogenesis in animal models

Tissue	Sample	Dose	Duration ^a	Tumor Incidence	Multiplicity
MNU-induced tumors	15	0.0	17	80%	1.6
In rats	15	50 μ g/kg diet	17	47% ^a	0.8 ^a
DMBA-induced tumors	20	0.0	22	85%	1.9
In rats	20	20 μ g/kg diet	22	40% ^a	1.3
UIISO-BCA-4 xenograft	5	0.0	6	100%	NA
In athymic mice	5	8 ng (s.c.) ^b	6	0% ^c	NA
UIISO-BCA-4 xenograft	5	0.0	6	100%	NA
In athymic mice	5	12.5 μ g/kg diet	6	0% ^a	NA
BT-474 xenograft	5	0.0	8.5	0.01 cm ³	NA
In athymic mice	5	12.5 μ g/kg diet	8.5	0.125 cm ^{3c}	NA

^a Duration in weeks^b 8 ng 1 α (OH)D5 subcutaneously injected thrice weekly for 60 days^c Significantly different from control ($p < 0.05$)^d Results are expressed as tumor volume (cm³)

nificantly reduced from control ($p=0.12$), whereas the high-dose group had a significantly lower tumor incidence ($p=0.03$). However, when the three groups were compared using log-rank analysis, the comparison reached statistical significance ($p=0.0495$). Tumor multiplicity was not significantly different between the control group and the 25- μ g/kg diet group, but it was significantly lower in the high-dose group ($p=0.02$).

The encouraging results from MNU-carcinogenesis model prompted us to extend our in vivo experiments. Since MNU is a direct-acting carcinogen, we chose another mammary-specific carcinogen that needs to be metabolized, such as DMBA. For the DMBA carcinogenesis study, 7-week-old rats (20 per group) were given 15 mg DMBA intragastrically. 1 α (OH)D5 was supplied in the diet (20–40 μ g/kg diet) 2 weeks prior to carcinogen treatment. The control group showed 85% tumor incidence and the high-dose group showed 60% incidence, while the low-dose group showed a significant decrease in incidence (40%). Table 3 summarizes the results from in vivo experiments. Although the high-dose group did not show a significant decrease in tumor incidence, it had significantly lower tumor multiplicity (0.6 compared to 1.9 in the control group). Moreover, the chemopreventive efficacy of 1 α (OH)D5 was more pronounced when provided at progression stages of the disease.

In addition to assessing chemopreventive properties of 1 α (OH)D5 in mammary carcinogenesis, we evaluated its efficacy as a possible chemotherapeutic agent. These experiments were carried out in xenograft models, as previously described (Mehta and Mehta 2002). Initial studies were conducted using xenograft of UIISO-BCA-4 cells pretreated with 1 μ M 1 α (OH)D5 for 10 days, which failed to form tumors in athymic (4-week-old) mice. In other studies, UIISO-BCA-4 cells were xenografted in athymic mice and either 8 ng 1 α (OH)D5 per animal was injected IP thrice a week or 1 α (OH)D5 was provided in the diet at

12.5 μ g/kg diet for 6 weeks. All the animals in the control group formed tumors whereas only one of the treated animals showed a scab-like structure at injection site in the IP group. Forty percent of controls showed metastasis to lymph nodes but 1α (OH)D₅ treatment prevented metastasis of cells transplanted in athymic mice (Mehta and Mehta 2002). In the dietary treatment group, 1α (OH)D₅ inhibited growth of UISO-BCA-4 cells and the tumor volume was suppressed to nearly 50% of control. Similar results were obtained with BT-474 xenograft in athymic mice. These results suggest that 1α (OH)D₅-induced cell growth inhibition and differentiation is protective against tumor growth in the xenograft model as well.

Growth Response of Normal versus Transformed Cells to 1α (OH)D₅

While we established that 1α (OH)D₅ has growth inhibitory action on cancer cells, the effects on normal breast epithelial cells were not known. In order to determine that, we cultured mammary glands from mouse with appropriate hormones in the absence of any carcinogens. Ten glands were treated with 1α (OH)D₅ and other glands were used as controls. At the end of 6-day culture, the glands were terminated, paraffin embedded, and sectioned for pathological evaluation. Histopathological examination showed no difference in the growth and morphology of glands treated with 1α (OH)D₅ from that of control glands. In view this result, we evaluated the effects of 1α (OH)D₅ on MCF-12F cells, which are nontumorigenic breast epithelial cells derived from reduction mammaplasty from a 60-year-old Caucasian woman. These cells were spontaneously immortalized by long-term culture in low- Ca^{++} media. To determine their growth response, MCF-12F cells were incubated with 1α (OH)D₅ for various intervals, but no growth inhibitory effect was observed at the 1- μ M concentration.

To establish selectivity of 1α (OH)D₅ effects on transformed or preneoplastic cells, we transformed MCF-12F cells with DMBA and MNU to study if the transformation status could affect the response to 1α (OH)D₅. Transformation was performed using the protocol described elsewhere (Lazzaro et al. 1997). Briefly, passage 10 MCF-12F cells were grown to subconfluency in tissue culture dishes and incubated with DMBA (2 μ g DMBA/ml culture media) for 24 h. The procedure was repeated the next day. Extensive cell death resulted. The surviving cells were allowed to grow in fresh medium and later selected out with serum starvation. The resulting cell line was designated MCF-12F_{DMBA}. Similarly, in another experiment, MNU was dissolved in acidified saline (pH 5.3) and added to subconfluent MCF-12F cells at a concentration of 25 μ g/ml twice daily for 2 days. The surviving cells were allowed to grow and the new cell line was established after serum starvation. These cells were called MCF-12F_{MNU}. The growth rate and morphological characteristics were compared between these cell lines. The growth rates of transformed cells were three times higher than MCF-12F. By the fifth passage of postcarcinogen treatment, the MCF-12F_{DMBA} doubling time was reduced to one-third of MCF-12F,

Table 4 Growth effects of 1 μ M 1 α (OH)D5 on normal and transformed MCF-12F breast epithelial cells

Cell line	Treatment	Cell count	Cell cycle (% G-1)	MTT absorbance
MCF-12F	Control	47,250 \pm 474	68	0.045 \pm 0.06
	1 α (OH)D5	45,820 \pm 587	71	0.044 \pm 0.04
MCF-12F _{MNU}	Control	91,800 \pm 120	43	0.185 \pm 0.06
	1 α (OH)D5	73,616 \pm 138*	65	0.078 \pm 0.01*
MCF-12F _{DMBA}	Control	105,470 \pm 42.4	49	0.128 \pm 0.02
	1 α (OH)D5	8,035 \pm 91*	67	0.075 \pm 0.01*

* Significantly different from control ($p < 0.05$)

while for MCF-12F_{MNU}, it was reduced to one-fourth of MCF-12F. Moreover, the transformed cell lines did not exhibit the contact inhibition characteristic of the normal cells.

As mentioned earlier, MCF-12F cells showed no growth inhibitory response with 1 α (OH)D5 treatment. The transformed cells, on the other hand, showed significant growth inhibition (60% for MCF-12F_{MNU} and 40% for MCF-12F_{DMBA}), as determined by the MTT absorbance assay. Other measures of growth provided similar results (Table 4). These studies indicate that the transformed cells respond differently to 1 α (OH)D5 treatment than the parent cell line.

Potential Mechanism of Action of 1 α (OH)D5 in Breast Cancer Prevention and Therapy

Previously mentioned studies have implicated the involvement of VDR in 1 α (OH)D5-mediated growth effects. VDR- highly metastatic cells such as MDA-MB-231 and MDA-MB-435 do not respond to 1 α (OH)D5 treatment. Moreover, mammary epithelial cells which lack VDRs also fail to respond to 1 α (OH)D5 and do not show induction of VDR and TGF- β 1 (Mehta et al. 1997a). VDR+ breast cancer cells, such as T-47D, had been shown to increase transcription of VDR upon incubation with 1 α (OH)D5 as determined by RT-PCR (Lazzaro et al. 2000). This VDR induction was not observed in the cell line BT-474, either at transcription or expression levels, upon treatment with 1 α (OH)D5. A possible explanation could be the high constitutive levels of VDR present in this cell line. To ascertain VDR-mediated VDRE transactivation activity of 1 α (OH)D5, we used the CAT reporter gene containing VDRE (VDRE-tk-CAT). For this purpose, CV-1 monkey renal cancer cells were used as these lack a functional VDR. After VDRE-tk-CAT transient transfection into CV-1 cells, 1 α (OH)D5 could not induce the CAT activity in these cells. But when the cells were cotransfected with VDRE and VDR, there was an enhanced expression of CAT activity, suggesting the capability of 1 α (OH)D5 to activate VDR-mediated signaling. The relative CAT activity in CV-1 cells that had been cotransfected with VDRE and VDR was 200,000-fold higher than control when treated with 0.1 μ M 1 α (OH)D5 (Lazzaro et al. 2000).

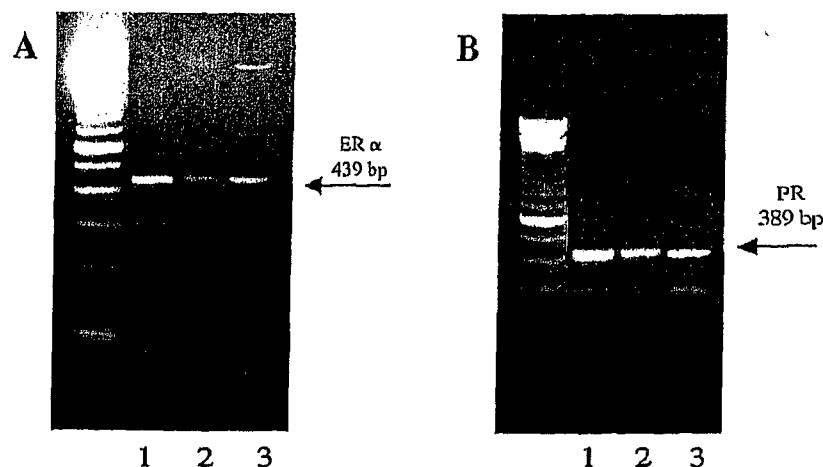


Fig. 4 Down-regulation of estrogen- (A) and progesterone- (B) receptor transcription with vitamin D₃ and its analog in BT-474 cells as determined by RT-PCR. Lane 1 control, lane 2 $1\alpha,25(\text{OH})_2\text{D}_3$, lane 3 $1\alpha(\text{OH})\text{D}_5$

Breast cancer UIISO-BCA-4 cells are ER⁻ and PR⁻, but VDR⁺. These cells responded differently to $1\alpha(\text{OH})\text{D}_5$ than the ER⁺ cells (Mehta et al. 2003). UIISO-BCA-4 cells were treated with 0.1 μM $1\alpha(\text{OH})\text{D}_5$ for 10 days. The $1\alpha(\text{OH})\text{D}_5$ treatment resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, $\alpha 2$ -integrin, nm23, and VDR, manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of $1\alpha(\text{OH})\text{D}_5$. While $1\alpha(\text{OH})\text{D}_5$ induced differentiation in ER⁻ cells, it induced apoptosis in ER⁺ BT-474 and MCF-7 cells, as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta et al. 2003). In both these cell lines, there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of $1\alpha(\text{OH})\text{D}_5$ differ in ER⁺ breast cancer cells, we examined the effects of $1\alpha(\text{OH})\text{D}_5$ on estrogen-dependent signaling in the ER⁺ PR⁺ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen-inducible PR transcription upon treatment with $1\alpha(\text{OH})\text{D}_5$, as determined by RT-PCR (Fig. 4). This was in turn followed by down-regulation at the expression level, as estimated by immunocytochemistry (Fig. 5). These results are consistent with reports by other researchers that describe the role of vitamin D₃ in down-regulation of estrogen-inducible genes (Swami et al. 2000; Stoica et al. 1999). The vitamin D₃-VDR pathway may be a negative feedback mechanism to regulate the estrogen-induced proliferation of the mammary tissue. Some researchers have postulated an interaction of VDR-D₃ to ERE to repress the estrogen-mediated gene transcription (Welsh et al. 1998; Demirpence et al. 1994).

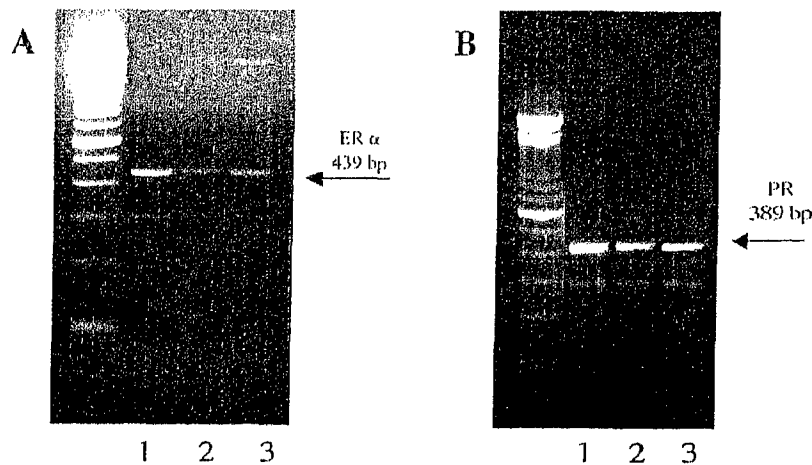
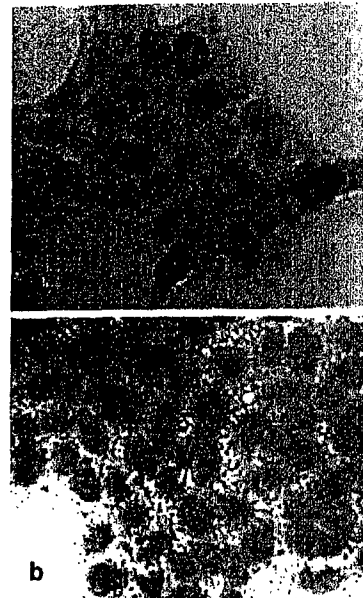


Fig. 4 Down-regulation of estrogen- (A) and progesterone- (B) receptor transcription with vitamin D₃ and its analog in BT-474 cells as determined by RT-PCR. Lane 1 control, lane 2 $1\alpha,25$ (OH) $_2$ D $_3$, lane 3 1α (OH)D $_5$

Breast cancer UIISO-BCA-4 cells are ER $-$ and PR $-$, but VDR $+$. These cells responded differently to 1α (OH)D $_5$ than the ER $+$ cells (Mehta et al. 2003). UIISO-BCA-4 cells were treated with 0.1 μ M 1α (OH)D $_5$ for 10 days. The 1α (OH)D $_5$ treatment resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, α 2-integrin, nm23, and VDR, manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of 1α (OH)D $_5$. While 1α (OH)D $_5$ induced differentiation in ER $-$ cells, it induced apoptosis in ER $+$ BT-474 and MCF-7 cells, as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta et al. 2003). In both these cell lines, there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of 1α (OH)D $_5$ differ in ER $+$ breast cancer cells, we examined the effects of 1α (OH)D $_5$ on estrogen-dependent signaling in the ER $+$ PR $+$ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen-inducible PR transcription upon treatment with 1α (OH)D $_5$, as determined by RT-PCR (Fig. 4). This was in turn followed by down-regulation at the expression level, as estimated by immunocytochemistry (Fig. 5). These results are consistent with reports by other researchers that describe the role of vitamin D $_3$ in down-regulation of estrogen-inducible genes (Swami et al. 2000; Stoica et al. 1999). The vitamin D $_3$ -VDR pathway may be a negative feedback mechanism to regulate the estrogen-induced proliferation of the mammary tissue. Some researchers have postulated an interaction of VDR-D $_3$ to ERE to repress the estrogen-mediated gene transcription (Welsh et al. 1998; Demirpence et al. 1994).

Fig. 5a, b Down-regulation of progesterone receptor (PR) expression with $1\alpha(\text{OH})\text{D}_5$ treatment in BT-474 cells as detected by immunocytochemical analysis. Percentage of cells stained positive for PR were 78% in control (a) and 46% in treated cells (b)



Since vitamin D_3 is known to regulate a wide variety of genes, we investigated other potential gene targets of $1\alpha(\text{OH})\text{D}_5$ in BT-474 cells. The microarray was performed using Human UniGene 1 by Incyte Genomics, Inc. (Palo Alto, CA, USA), which contained 8,000 genes along with appropriate controls. Among the major targets of $1\alpha(\text{OH})\text{D}_5$ were the estrogen-inducible genes PR, trefoil factor 1 (pS2), and trefoil factor 3 ($p < 0.05$). A few selected genes that were statistically significantly altered are presented in Table 5.

As mentioned earlier, the transformed MCF-12F cells showed growth inhibition even though these cells express very low levels of steroid receptors. It is possible that other mechanisms are at work to bring about growth arrest in MCF-12F_{DMBA} and MCF-12F_{MNU} cells. Therefore, we used Clontech Atlas microarrays (Genomics Inc.) with 10,000 genes to identify differentially expressed genes in the transformed MCF-12F_{MNU} cells as compared to the MCF-12F parent cell lines. In a second comparison, we assessed the genes differentially expressed by $1\alpha(\text{OH})\text{D}_5$ treatment in MCF-12F_{MNU} cells. Interestingly, many genes that were differentially expressed in MCF-12F_{MNU} cells compared to the MCF-12F cells were altered inversely in $1\alpha(\text{OH})\text{D}_5$ treated MCF-12F_{MNU} cells (Table 5). Most of the genes that were affected were transcription-related and mitochondrial genes. Of interest are proteins such as vimentin, prohibitin, MAPK-7, and HSP-27, which are usually expressed at higher levels in mammary tumors (Atanaskova et al. 2002; Zajchowski et al. 2001; Storm et al. 1996). These proteins were down-regulated in $1\alpha(\text{OH})\text{D}_5$ -treated cells. Differentiation-related proteins such as integrins and cadherins were up-regulated by $1\alpha(\text{OH})\text{D}_5$ in both BT-474 and MCF-12F_{MNU} cell systems.

Table 5 Microarray analysis to determine effects of 1 μ M 1 α (OH)D5 and MNU-induced transformation on selected genes

Comparison	Genes up-regulated	Genes down-regulated
BT-474 (control) \pm 1 α (OH)D5 Incyte Genomics Human UniGene 1 (8 K)	Cytochrome P450 (vitamin D ₃ 24-hydroxylase) Caspase 3 Cadherin 18 type 2	Trefoil factor 1 (pS2) Progesterone receptor Trefoil factor 3 MMP-9 Thymidine kinase 2 (mitochondrial) Transcobalamin E2F-4 Integrins Glutathione peroxidase 4 Ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1 Prohibitin Vimentin MAPK-7 Thioredoxin HSP-27
MCF-12F (control) vs. MCF- 12F _{MNU} Clontech Atlas Arrays (10 K)	TGF α Prohibitin Calpain 4 Pituitary tumor transforming 1 HSP-27 Thioredoxin Keratin 6A and 6B	
MCF-12F _{MNU} (control) \pm 1 α (OH)D5 Clontech Atlas Arrays (10 K)	Glutathione peroxidase 4 ornithine decarboxylase antizyme 1 Cystatin B Tissue inhibitor of metalloproteinase 1 TCTP-1 Integrin β 4 Cadherin 3 Cathepsin D	

Prohibitin might be a potentially important vitamin D₃-regulated protein, which was found to be more highly expressed in the transformed MCF-12F cells than the parent cell line (data not shown). Some studies have shown high prohibitin levels in tumor tissue and cancer cell lines (Jupe et al. 1996; Asamoto and Cohen, 1994). However, the role of this mitochondrial protein is controversial. Wang and co-workers (1999) have shown its involvement in regulation of the cell cycle, whereas others have shown that the levels do not represent the cell cycle-related functions but rather are indicative of mitochondrial stress (Coates et al. 2001). It is possible that the mitochondrial stress may be indicative of the higher proliferative rates of the transformed cells. Another protein of interest was thioredoxin, which was up-regulated in MCF-12F_{MNU} cells and down-regulated by 1 α (OH)D5 treatment. Thioredoxin is a redox protein with growth factor activity that modulates the activity of several proteins important for cell growth. Some researchers have observed increased thioredoxin transcription and expression in primary human tumors (Matsutani et al. 2001; Berggren et al. 1996). Administration of inhibitors of thioredoxin system has been shown to have antitumor activity in vivo (Kirkpatrick et al. 1999). Moreover, Gallegos and co-workers (1996) reported that transfec-

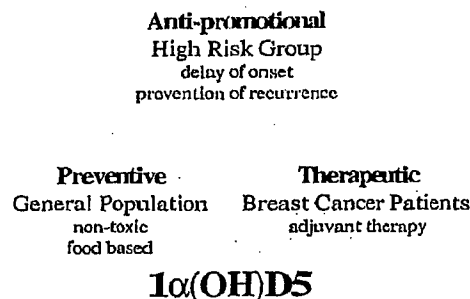


Fig. 6 Potential application of 1α(OH)D5 in breast cancer prevention and therapy

tion of dominant-negative mutant thioredoxin resulted in reversal of transformed phenotype of human breast cancer cells. Therefore, it appears that the mechanism of action of 1α(OH)D5 involves multiple genes and pathways, some of which have not yet been thoroughly investigated. Further studies are needed to elucidate the mechanism of action of 1α(OH)D5 in normal and cancer breast cells.

Conclusions

Results presented in this report on effects of 1α(OH)D5 are suggestive of its promise in chemoprevention. 1α(OH)D5 has consistently been shown to be effective in inhibiting growth of cancer cells as well as preneoplastic lesions in mammary glands in vitro. The in vitro effects are manifested in vivo as well. In the animal carcinogenesis models, 1α(OH)D5 had reduced the incidence of tumors as well as tumor multiplicity, and increased the latency period. Yet there were no changes in total body weight and no apparent signs of toxicity at efficacious doses. More recently, we completed preclinical toxicity studies in rats and dogs under good laboratory practices and regulations, providing an estimation of maximum tolerable dose. The concentration of 1α(OH)D5 required to achieve optimal cell regulatory effects is 100 times higher than the concentration of vitamin D₃. However, there is no hypercalcemia observed at this dose of 1α(OH)D5 to warrant concern. The mechanism of action of 1α(OH)D5 seems to involve VDR as well as cross-talk with the estrogen signaling pathway. It has been shown to inhibit estrogen-induced proliferation. Because of these properties, 1α(OH)D5 might prove suitable in a variety of applications. Furthermore, the differential gene expression profile clearly suggested that the effects of 1α(OH)D5 involve multiple pathways and genes, some of which have not yet been critically studied.

A scheme of possible applications of 1α(OH)D5 is presented in Fig. 6. From a prevention point of view, 1α(OH)D5 might be used in populations that are at high risk or to prevent or delay recurrence of breast tumors in breast cancer

patients. It might also be used in conjunction with other treatments for cancer therapy. Further studies are underway in our laboratory to determine if indeed 1 α (OH)D₃ would become available for clinical use in the future.

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Chemoprevention of mammary carcinogenesis by 1 α -hydroxyvitamin D₅, a synthetic analog of Vitamin D

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Abstract

Numerous analogs of Vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of Vitamin D without causing any toxicity. We synthesized such an analog, 1 α -hydroxy-24-ethylcholecalciferol [1 α -hydroxyvitamin D₅ or 1 α (OH)D₅], and showed that it was tolerated by rats and mice at a much higher dose than 1 α ,25 dihydroxy cholecalciferol [1 α ,25(OH)₂D₃]. This property makes it a prime candidate for chemoprevention studies. In the mouse mammary gland organ culture (MMOC), 1 α (OH)D₅ inhibited carcinogen-induced development of both mammary alveolar and ductal lesions. In vivo carcinogenesis study showed statistically significant reduction of tumor incidence and multiplicity in *N*-methyl-*N*-nitrosourea (MNU)-treated rats that were fed 25–50 μ g 1 α (OH)D₅/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1 α (OH)D₅ would be selective in suppressing proliferation of transformed cells, its effects on cell growth and proliferation were compared between BT474 (cancer) and MCF12F (non-tumorigenic) human breast epithelial cells. Results showed that 1 α (OH)D₅ induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. In addition, in MMOC it had no growth inhibitory effects on normal epithelial cell proliferation in the absence of carcinogen. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1 α (OH)D₅, whereas treatment with 1 α (OH)D₅ induced cell death in the explants of cancer tissue. These results collectively indicate that 1 α (OH)D₅ selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1 α (OH)D₅ were observed in Vitamin D receptor positive (VDR⁺) breast cancer cells, but not in highly metastatic VDR[−] breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1 α (OH)D₅ action may be mediated, in part, by VDR.

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Keywords: Vitamin D; Mammary carcinogenesis; Chemoprevention

1. Introduction

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process

by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent [1,2]. In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal

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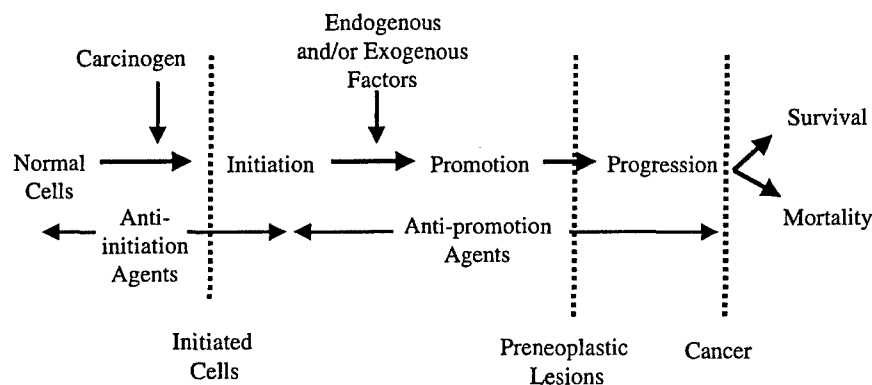


Fig. 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

cell to a transformed cancer cell is summarized in Fig. 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although the initiated cells have the potential to develop into malignant cancer, they may or may not form a tumor depending upon exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can advance to a preneoplastic stage leading progressively to malignancy. The chemopreventive agents that suppress the early events in transformation, such as preventing the mutagenic action of chemicals or other factors, are referred to as anti-initiation agents. On the other hand, chemicals that prevent further progression of initiated cells into transformed ones are termed anti-promotional agents [3,4]. Numerous classes of chemopreventive agents have been reported in the literature, including retinoids, deltanoids, cyclooxygenase inhibitors, inhibitors of polyamine and prostaglandin biosynthesis, lignans, calcium channel blockers, anti oxidants, etc. [5–7]. In this report, we have summarized the chemopreventive properties of a newly evaluated Vitamin D analog, 1- α -hydroxy-24-ethyl-cholecalciferol [$1\alpha(\text{OH})\text{D}_5$].

It has been well established that the active metabolite of Vitamin D, 1,25-dihydroxyvitamin D_3 , [$1,25(\text{OH})_2\text{D}_3$] is a steroid hormone and it exhibits potent cell-differentiating properties in leukemia cells as well as other cancer cells of epithelial origin [8,9]. The antiproliferative and differentiation-inducing effects of $1,25(\text{OH})_2\text{D}_3$ could be of clinical signifi-

cance in prevention or treatment of cancer of several target organs [10]. However, one major limitation in its clinical application is the fact that the efficacious concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ are cytotoxic [11]. The effective growth inhibitory concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ induces dangerously high levels of serum calcium resulting in loss of body weight and soft tissue calcification, which could be lethal [12]. This has resulted in generation of several non-toxic but antiproliferative synthetic analogs of the Vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated for their ability to suppress cancer cell growth in culture as well as in vivo models [13].

Typically, the structure of Vitamin D is divided into four parts (Fig. 2): ring A, open ring B, ring CD, and the side chain. Modifications can be made at all four sites, but the alteration of the ring CD is not common due to its rigid structure. Most alterations have been made at the open side chain. Nearly 800 analogs of Vitamin D have been synthesized so far, and about 300 of them have been evaluated in in vitro and in vivo experimental models [14,15]. Historically, a comparison of the toxicological profile of the Vitamin D series of compounds, including D_2 – D_6 , had suggested that D_5 was the least toxic of the D series of compounds [16]. In order to generate an effective but non-calcemic and non-toxic Vitamin D analog, we synthesized $1\alpha(\text{OH})\text{D}_5$ [17]. The structure of $1\alpha(\text{OH})\text{D}_5$ is shown in Fig. 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic

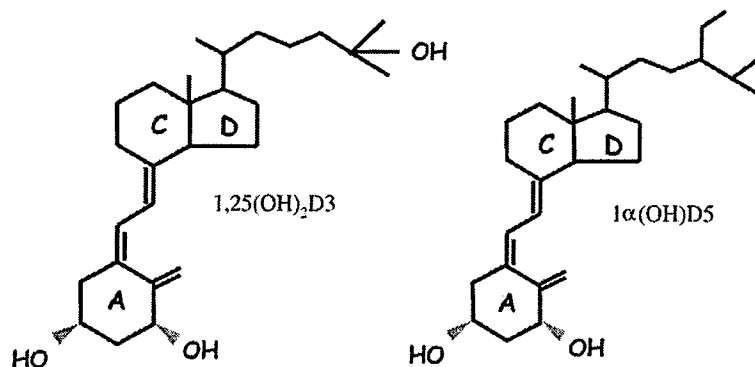


Fig. 2. Structural representation of 1,25(OH)₂D₃ and its analog 1α(OH)D₅.

pathway involves its association with high-affinity specific Vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors [18–20]. This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs [21], in addition to the previously known target organs that included intestine, kidney, and bone.

The VDR mRNA is about 4.6 kb, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10–100 fmoles/mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with Vitamin D response elements (VDRE) [22]. The natural metabolite 1α,25(OH)₂D₃ transactivates VDRE in VDR⁺ cells but fails to show interaction in VDR[−] cells. Hence, Vitamin D analogs that are able to transactivate VDR–VDRE are mainly mediating their action via genomic pathways. Non-genomic Vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of Vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis of calcium-bearing vesicles from lysosomes. The non-genomic pathway for Vitamin D action has been extensively reviewed elsewhere [23,24]. For this article, we have listed the chemopreventive properties and possible mode of action of 1α(OH)D₅.

2. Materials and methods

2.1. Cell lines

We purchased from the American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations the following cell lines: (1) the non-tumorigenic, estrogen receptor-negative (ER[−]), progesterone receptor-negative (PgR[−]), and low VDR breast epithelial cell line MCF12F; (2) ER⁺, PgR⁺, and VDR⁺ breast cancer cell lines BT474 and MCF7; and (3) ER[−], PR[−], and VDR[−] breast cancer cell lines MDA-MB-231 and MDA-MB-435.

2.2. Mouse mammary gland organ culture (MMOC)

The detailed procedures for culturing mammary glands from Balb/c mice have been previously reported in the literature [17,25] and outlined in Fig. 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's MB752/1 medium under 95% O₂ and 5% CO₂ at 37°C. The glands respond to growth-promoting hormones insulin, prolactin, aldosterone, and hydrocortisone and differentiate into distinct alveolar structures. Exposure of glands to 7,12-dimethylbenz(a)anthracene (DMBA) for 24 h on day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the

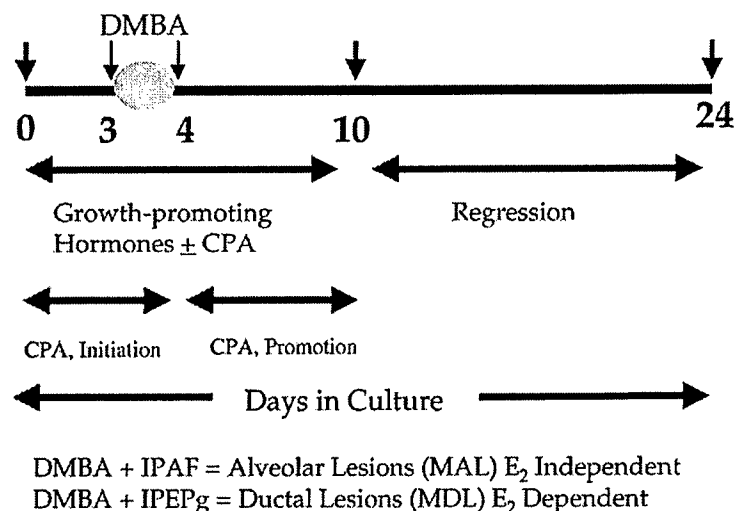


Fig. 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

glands develop mammary ductal lesions (MDL) with DMBA treatment [26]. We performed a dose response study to compare the effects of $1\alpha(\text{OH})\text{D}_5$ on MAL and MDL. Mammary lesions developed in the absence of $1\alpha(\text{OH})\text{D}_5$ served as controls. Additionally, we determined the effects of $1\alpha(\text{OH})\text{D}_5$ on normal mammary glands, where the glands were incubated with growth-promoting hormones and $1\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained, and analyzed for morphological characteristics and cell growth and compared with the appropriate controls.

2.3. Cell cycle analysis by flow cytometry

To determine cell cycle, we used flow cytometric analysis as described by Vindeløv et al. [27]. Breast epithelial non-tumorigenic and cancer cells were detached by trypsinization and were harvested. The cells were washed twice with PBS and pelleted. The pellet was resuspended and fixed in 85% ice-cold ethanol. After fixing, the cells were centrifuged and resuspended in citrate buffer and then incubated with NP-40, trypsin, and spermine for 15 min. This was followed by incubation with trypsin inhibitor and RNAase A. The cells were then stained with 0.04% propidium iodide solution. Approximately

10,000 cells were analyzed for DNA content using a Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle. Each experiment was repeated twice and student's *t*-test was used to assess differences.

2.4. Apoptosis

Programmed cell death was evaluated using acridine orange staining. Briefly, a $50\mu\text{l}$ suspension of breast epithelial cells was stained with $2\mu\text{l}$ of acridine orange/ethidium bromide solution ($100\mu\text{g/ml}$ acridine orange and $100\mu\text{g/ml}$ ethidium bromide in PBS). Cells were layered on a glass slide and examined under a fluorescent microscope with a $40\times$ objective lens using a fluorescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

2.5. Mammary carcinogenesis

The procedure for induction of mammary adenocarcinomas by *N*-methyl-*N*-nitrosourea (MNU) in Sprague-Dawley female rats has been described in detail previously [28] and is illustrated in Fig. 4. Briefly, 100-day-old female Sprague-Dawley rats

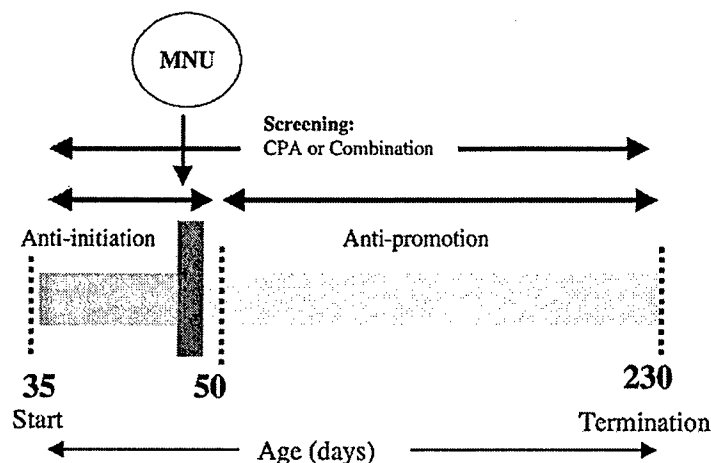


Fig. 4. Schematic diagram to show in vivo model of chemoprevention in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague–Dawley rats. CPA: chemopreventive agent.

were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or $1\alpha(\text{OH})\text{D}_5$ supplemented as 25 or 50 $\mu\text{g}/\text{kg}$ diet. Animals were sacrificed after 230 days of treatment. Mammary tumors were identified by palpation as well as necroscopy. Results were reported as effects of $1\alpha(\text{OH})\text{D}_5$ on the incidence, multiplicity, and latency of tumor development, and data were subjected to appropriate statistical analyses.

2.6. Effects of $1\alpha(\text{OH})\text{D}_5$ on normal and malignant breast tissue

Breast tissues were obtained from women undergoing mastectomy or lumpectomy. Explants were maintained in MEME medium, containing 5% stripped fetal bovine serum. The effects of $1\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. The effects of $1\alpha(\text{OH})\text{D}_5$ on cell morphology and Ki 67 were compared between the normal and adjacent cancer tissue from the same patient.

2.7. Statistical analysis

Statistical analyses were performed using Graph-Pad Instat[®] 3.0 software. All MMOC as well as MNU-induced carcinogenesis data were evaluated using χ^2 analysis. Cell viability, apoptosis, and cell

cycle results were assessed using two-tailed student's *t*-test with type I error set at 0.05. Serum calcium and phosphorus data were tested with student's *t*-test as well. All in vitro experiments were performed in duplicates and repeated twice.

3. Results and discussion

3.1. Synthesis and toxicity of $1\alpha(\text{OH})\text{D}_5$

Nearly 300 analogs of $1,25(\text{OH})_2\text{D}_3$ have been evaluated in various experimental systems in the hope of generating analogs that are more efficacious with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs include EB1089 [29], KH1060 [30], R024-5531 [31], and 22-Oxacalcitriol [32], which are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of $1,25(\text{OH})_2\text{D}_3$, R024-5531, has no calcemic activity, while other analogs do express dose-related calcemia [33,34]. Since it had been reported previously that Vitamin D_5 is the least toxic series of Vitamin D compounds, we synthesized $1\alpha(\text{OH})\text{D}_5$ with the intention of testing its chemopreventive potential. The chemical synthesis of $1\alpha(\text{OH})\text{D}_5$ has been previously reported from our laboratory [17].

Since calcemic activity is an obstacle to the development of effective Vitamin D analogs suitable for clinical use, we determined serum calcium and phosphorous concentrations after treating Vitamin D-deficient rats with $1,25(\text{OH})_2\text{D}_3$ and $1\alpha(\text{OH})\text{D}_5$. As reported earlier, male Sprague–Dawley rats (8–10 per group) were fed Vitamin D-deficient diet for 3 weeks, and baseline serum calcium levels were determined. Rats showing $<6\text{ mg/dl}$ serum calcium were given $1\alpha(\text{OH})\text{D}_5$ for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that $1,25(\text{OH})_2\text{D}_3$ significantly ($P < 0.001$) increased serum calcium concentration at a daily dose of $0.042\text{ }\mu\text{g/kg}$ diet, whereas there was no elevation in serum calcium levels among $1\alpha(\text{OH})\text{D}_5$ -treated animals [17].

A similar experiment was carried out using Vitamin D-sufficient regular diet. Female Sprague–Dawley rats were treated with various concentrations of $1,25(\text{OH})_2\text{D}_3$ (0.8 – $12.8\text{ }\mu\text{g/kg}$ diet) and $1\alpha(\text{OH})\text{D}_5$ (6.4 – $50\text{ }\mu\text{g/kg}$ diet) for 2 months. Calcium concentration was increased by $1,25(\text{OH})_2\text{D}_3$ treatment, while no serum calcium elevation was observed in $1\alpha(\text{OH})\text{D}_5$ -treated ($25\text{ }\mu\text{g/kg}$ diet) animals (Table 1). There was no effect on the final body weight at any dose of $1\alpha(\text{OH})\text{D}_5$ used in this study. These results indicate that $1\alpha(\text{OH})\text{D}_5$ is considerably less toxic compared to the natural hormone.

More recently, we completed an extensive preclinical toxicity study in both sexes of rats and dogs under good laboratory practice (GLP). Results showed that dogs are relatively more sensitive to the higher

dose of $1\alpha(\text{OH})\text{D}_5$ than are rats. We concluded from those studies that $1\alpha(\text{OH})\text{D}_5$ is calcemic in dogs at concentrations higher than $10\text{ }\mu\text{g/kg}$ diet. The non-calcemic analog R024-5531 shows toxicity in rats without having an effect on serum calcium concentrations. On the other hand, $1\alpha(\text{OH})\text{D}_5$ can be tolerated at a higher concentration without other toxicity outcomes.

Chemoprevention of mammary carcinogenesis by $1\alpha(\text{OH})\text{D}_5$: The chemopreventive properties of $1\alpha(\text{OH})\text{D}_5$ have been evaluated in two experimental systems in our laboratory. These include MMOC and MNU-induced mammary carcinogenesis in Sprague–Dawley rats. Mouse mammary glands respond to DMBA and develop preneoplastic mammary alveolar as well as ductal lesions in organ culture. As shown in Fig. 3, the efficacy of a potential chemopreventive agent can be assessed in this assay. If the agent is present and effective prior to carcinogen treatment, its effects are considered as anti-initiation, whereas, if it is effective subsequent to carcinogen, then its effect are anti-promotional. Both types of effects can be determined using the MMOC model.

We showed previously that $1\alpha(\text{OH})\text{D}_5$ inhibits the development of mammary lesions in a dose-responsive manner [17]. However, it requires 10-fold higher concentration than the effective concentration of $1,25(\text{OH})_2\text{D}_3$. The most effective dose of $1,25(\text{OH})_2\text{D}_3$ in suppressing $>60\%$ incidence of MAL is 10^{-7} M , while $1\alpha(\text{OH})\text{D}_5$ is equally effective at 10^{-6} M without showing cytotoxicity. We also evaluated $1\alpha(\text{OH})\text{D}_5$ effects in the MDL model [25]. The results are summarized in Fig. 5. We found $1\alpha(\text{OH})\text{D}_5$ to be equally effective against alveolar and ductal lesions.

Since most of the effects of Vitamin D are mediated through VDR, we determined VDR induction by $1\alpha(\text{OH})\text{D}_5$ in MMOC as well as in breast cancer cell lines [17]. There was a significant increase in the expression of VDR in the epithelial cells of MMOC as determined by immunocytochemistry. Additionally, $1\alpha(\text{OH})\text{D}_5$ also upregulated the expression of TGF β in the epithelial cells of MMOC [15].

Based on these results, it was reasonable to expect chemopreventive activity of $1\alpha(\text{OH})\text{D}_5$ in an in vivo model. Prior to conducting in vivo carcinogenesis studies, a dose tolerance study was conducted in Sprague–Dawley rats. Animals were provided with increasing concentrations of $1\alpha(\text{OH})\text{D}_5$, ranging from

Table 1
Effects of $1\alpha(\text{OH})\text{D}_5$ treatment on serum calcium and phosphorous levels in Sprague–Dawley rats ($n = 10$)

Agent	Dose ($\mu\text{g/kg}$)	Serum Ca (mg/dl)	Serum P (mg/dl) ^a	BW (% gain)
None		6.3	3.6	100
$1,25(\text{OH})_2\text{D}_3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5*	8.9	70*
$1\alpha(\text{OH})\text{D}_5$	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

* Significantly different from control ($P < 0.05$).

^a Significance not determined.

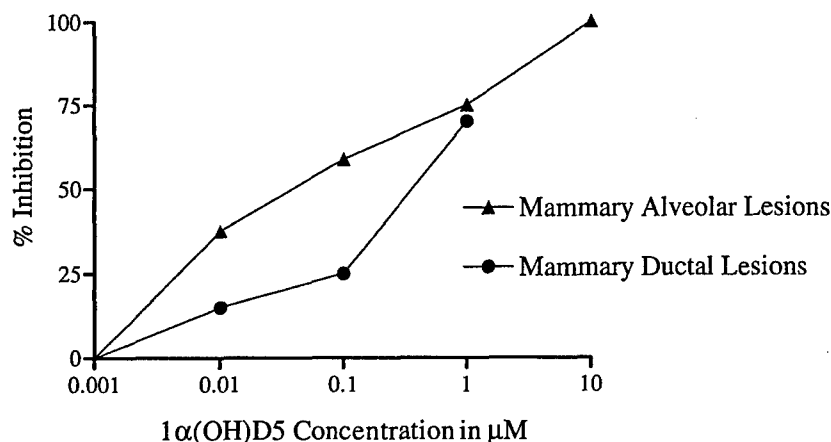


Fig. 5. Effect of $1\alpha(\text{OH})\text{D}_5$ on mouse mammary organ culture (MMOC). The glands were incubated with $1\text{ }\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Fifteen glands were used per group. A difference in inhibition of greater than 60% was considered significant ($P < 0.05$, χ^2). Data shows significant inhibition of preneoplastic MAL and MDL with $1\alpha(\text{OH})\text{D}_5$ treatment.

1 to 100 $\mu\text{g/kg}$ diet for 6 weeks. The animals did not show any adverse effects at any concentration of $1\alpha(\text{OH})\text{D}_5$, while the natural hormone was toxic at 3.5 $\mu\text{g/kg}$ diet.

For the MNU-induced mammary carcinogenesis studies, animals were fed $1\alpha(\text{OH})\text{D}_5$ at 25 and 50 $\mu\text{g/kg}$ diet for 3 months. The experimental diet was given to the animals 1 week prior to the carcinogen treatment and continued until the end of the study. Results are shown in Table 2. The results indicated a dose-dependent suppression of tumor incidence by $1\alpha(\text{OH})\text{D}_5$. This was accompanied by a reduction in tumor multiplicity and an increase in tumor latency [28]. These results are comparable with those of EB1089, R024-5531, and KH1060. The *in vivo* results as well as the results from MMOC clearly suggest a potential for $1\alpha(\text{OH})\text{D}_5$ to be developed as a chemopreventive and therapeutic agent.

Table 2
Chemoprevention of MNU-induced mammary carcinogenesis by $1\alpha(\text{OH})\text{D}_5$ in rats

Treatment	Dose ($\mu\text{g/kg}$)	<i>n</i>	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
$1\alpha(\text{OH})\text{D}_5$	25	15	53*	1.2	230
$1\alpha(\text{OH})\text{D}_5$	50	15	47*	0.8*	226

* Significantly different from control ($P < 0.05$).

3.2. Selectivity of $1\alpha(\text{OH})\text{D}_5$ action for transformed cells

We compared the growth effects of $1\alpha(\text{OH})\text{D}_5$ in various steroid receptor-positive as well as negative breast epithelial cell lines. These cell lines included (1) non-tumorigenic MCF12F breast epithelial cells, (2) ER^+ , PgR^+ , VDR^+ , BT474, and MCF7 cells, and (3) ER^- , PgR^- , and VDR^- highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both $1,25(\text{OH})_2\text{D}_3$ and $1\alpha(\text{OH})\text{D}_5$ were efficacious in suppressing cell proliferation of ER^+ , PR^+ , and VDR^+ BT474, T47D, ZR75, and MCF7 breast cancer cells. These compounds induced differentiation of ER^- , PgR^- , VDR^+ , and BCA-4 cells [35] but did not show any growth effects in MDA-MB-435 and MDA-MB-231 cells. Other researchers have also reported similar results with other Vitamin D analogs [36]. Although our results indicate that the presence of VDR is necessary to potentiate Vitamin D's effect, it does not explain the lack of Vitamin D's effect on MCF12F cells that express low levels of VDR.

In order to examine whether $1\alpha(\text{OH})\text{D}_5$ selectively inhibits cell proliferation in transformed cells only, we evaluated the effects of $1\alpha(\text{OH})\text{D}_5$ on non-tumorigenic breast epithelial cells and compared them to the effects on BT474 breast cancer cells.

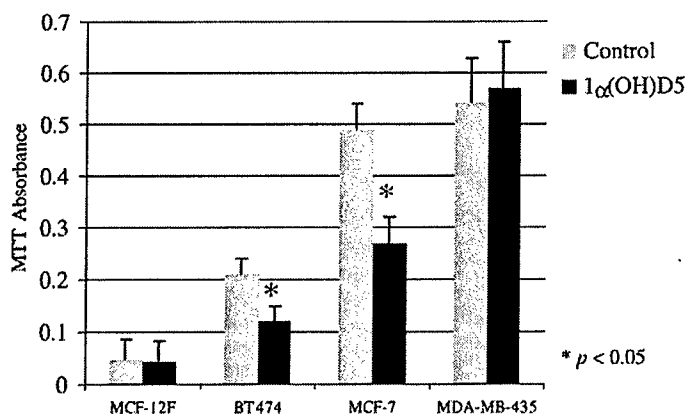


Fig. 6. Effects of $1\alpha(\text{OH})\text{D}_5$ on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with $1\text{ }\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for 2 days and incubated with MTT for 2 h. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells. Each experiment was repeated twice and differences between the mean were assessed using student's *t*-test.

As shown in Fig. 6, incubation of MCF12F breast epithelial cells for 6 days with $1\alpha(\text{OH})\text{D}_5$ at $1\text{ }\mu\text{M}$ concentration did not result in suppression of cell proliferation as determined by the MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with $1\alpha(\text{OH})\text{D}_5$ treatment. These results suggested that the effect of Vitamin D analog might be selective for transformed cells. The antiproliferative effects of $1\alpha(\text{OH})\text{D}_5$ were also evident in in vivo experiments. Xenograft of ER⁺, PgR⁺, VDR⁺, MCF7, ZR75/1, and BT474 cells or ER⁻, PgR⁻, VDR⁺, and BCA-4 cells responded to $12.5\text{ }\mu\text{g}$ $1\alpha(\text{OH})\text{D}_5/\text{kg}$ diet and showed suppressed growth of these cells in athymic mice [35].

To confirm the selectivity of $1\alpha(\text{OH})\text{D}_5$ for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared the efficacy of $1\alpha(\text{OH})\text{D}_5$ between MCF12F cells with that of MNU-transformed MCF12F (MCF12F_{MNU}) cells. The MCF12F_{MNU} cells have recently been established in our laboratory (unpublished data). The MCF12F_{MNU} cells have altered morphology and growth properties as well as different growth factor requirements (Hussain and Mehta, unpublished data). Incubation of MCF12F and MCF12F_{MNU} with $1\text{ }\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for 6 days resulted in 50% growth inhibition in MCF12F_{MNU} cells without having any significant effects on MCF12F growth.

In a second study using the MMOC model, the effects of $1\alpha(\text{OH})\text{D}_5$ were determined in mammary glands. Mammary glands respond to growth-promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with $1\text{ }\mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for 6 days did not affect the growth-promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen, and progesterone (Fig. 7). Contrarily, $1\alpha(\text{OH})\text{D}_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Fig. 5).

Experiments to determine the selectivity of $1\alpha(\text{OH})\text{D}_5$ action against transformed cells were further extended to human tissues. The effects of $1\alpha(\text{OH})\text{D}_5$ on the explants derived from normal breast tissues were compared with those of cancer tissue. Breast tissue samples were obtained from women undergoing mastectomy or lumpectomy at the University of Illinois at Chicago Hospital. Tissue explants of tumors and normal adjacent cells were incubated for 72 h in the MEME containing 5% fetal calf serum with or without $1\alpha(\text{OH})\text{D}_5$ at $1\text{ }\mu\text{M}$ concentration. Tissue sections were histopathologically evaluated, and Ki 67 expression was determined. Results showed that the histopathology of control and $1\alpha(\text{OH})\text{D}_5$ -treated normal breast tissue was identical with no difference in apoptosis or Ki 67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with

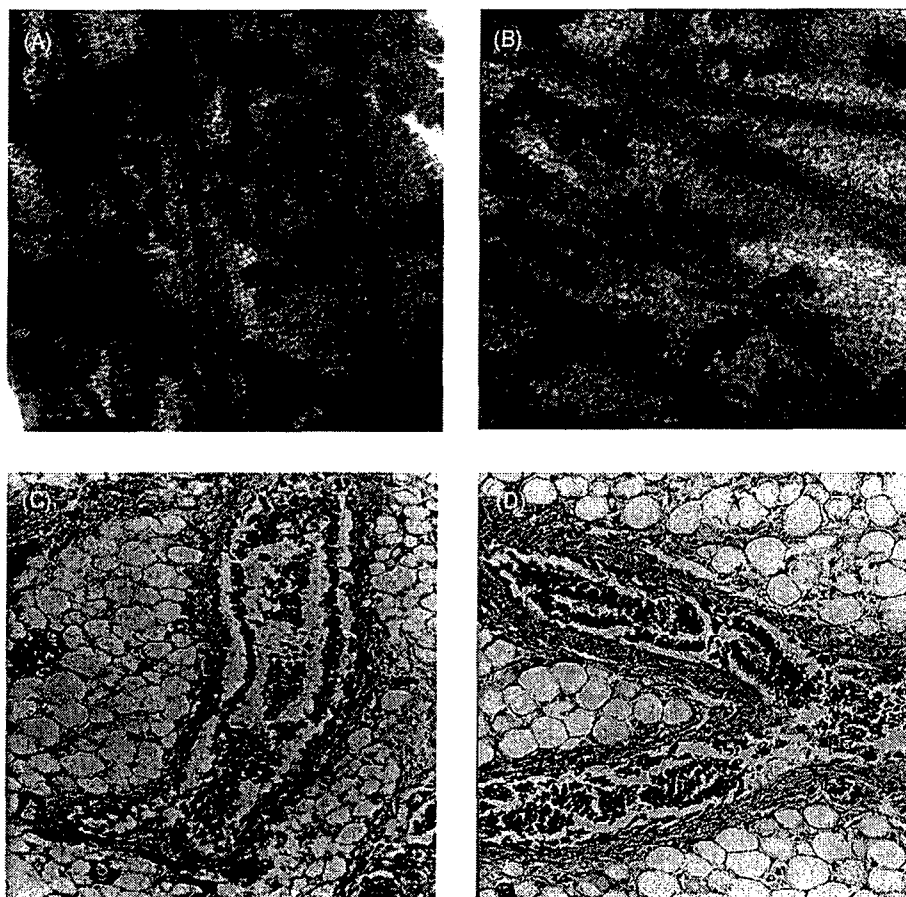


Fig. 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and $1\alpha(\text{OH})\text{D}_5$ treated glands. (A) control; (B) $1\alpha(\text{OH})\text{D}_5$; fixed and stained with carmine; (C) control and (D) $1\alpha(\text{OH})\text{D}_5$; fixed, sectioned, and stained with H and E.

condensed chromatin and reduced Ki 67 expression after 72-h incubation with $1\alpha(\text{OH})\text{D}_5$ (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, $1\alpha(\text{OH})\text{D}_5$ is selective for its effects on pre-cancerous or cancer cells but shows no effect on normal breast epithelial cell growth.

3.3. Mechanism of $1\alpha(\text{OH})\text{D}_5$ action

The effects of $1\alpha(\text{OH})\text{D}_5$ have also been evaluated in several breast cancer cell lines [37]. Although these studies do not focus directly on chemoprevention, they do provide excellent insight into the mechanism of action of $1\alpha(\text{OH})\text{D}_5$ and its efficacy as an

anti-proliferative agent. We had reported that, in ER^+ , PgR^+ , breast cancer cells, $1\alpha(\text{OH})\text{D}_5$ inhibited cell growth by inducing apoptosis as well as differentiation, whereas in ER^- but VDR^+ cells, it induced cell differentiation without the induction of apoptosis [35]. Similar results have also been reported by numerous investigators using other analogs of Vitamin D [38]. The data from these studies consistently reported that breast cancer cells expressing VDR respond to Vitamin D analogs. These results suggested that the mode of action of $1\alpha(\text{OH})\text{D}_5$ depended not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of $1\alpha(\text{OH})\text{D}_5$ on cell cycle were determined using breast cancer cells. The BT474 cells

Table 3
Effects of $1\alpha(\text{OH})\text{D}_5$ on cell cycle phases in breast epithelial cell lines

Types	G1 (%)	S (%)	G2 (%)	G1/G2 (%)
BT474				
Control	60.7	30.5	8.8	6.9
$1,25(\text{OH})_2\text{D}_3$	71.6*	22.1	6.3	11.4
$1\alpha(\text{OH})\text{D}_5$	85.7*	10.3	4.0	21.4
MCF7				
Control	61.2	28.6	10.1	6.1
$1,25(\text{OH})_2\text{D}_3$	71.9*	19.3	8.8	8.2
$1\alpha(\text{OH})\text{D}_5$	70.0*	20.4	9.6	7.3
MDAMB435				
Control	22.8	31.3	45.9	0.5
$1,25(\text{OH})_2\text{D}_3$	21.1	33.0	45.3	0.5
$1\alpha(\text{OH})\text{D}_5$	21.1	23.6	55.3	0.4
MCF12F				
Control	72.4	16.2	11.4	6.4
$1,25(\text{OH})_2\text{D}_3$	61.1*	20.2	19.0	3.2
$1\alpha(\text{OH})\text{D}_5$	67.3*	16.2	16.5	4.1

* Significantly different from control ($P < 0.05$).

were treated with $1\ \mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$ for various time points and processed for FACS analysis. Results showed that 70% of the control cells were distributed in the G1 phase, whereas treatment with $1\alpha(\text{OH})\text{D}_5$ induced growth arrest with 84% cells in the G1 phase of the cycle. The results are summarized in Table 3. In agreement with our cell proliferation data, there was no difference between the distribution of cells in various cell cycle stages for MCF12F and MBA-MD-231 cells with $1\alpha(\text{OH})\text{D}_5$ treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid

of steroid receptors; therefore, these cells were not expected to respond to $1\alpha(\text{OH})\text{D}_5$ treatment. These results further confirm that the action of $1\alpha(\text{OH})\text{D}_5$ may be mediated, in part, by VDR.

The mechanism of action of $1\alpha(\text{OH})\text{D}_5$ was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(\text{OH})_2\text{D}_3$ or $1\alpha(\text{OH})\text{D}_5$ for 72 h and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Fig. 8 shows that BT474 cells underwent apoptosis with $1\alpha(\text{OH})\text{D}_5$ treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells from those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^- , PgR^- , VDR^+ , BCA-4 cells, though there was an induction of differentiation as shown by casein, lipids, and $\alpha 2$ integrin expression [35].

Chemopreventive agents are being developed mostly for people who do not yet have disease but are at high risk of developing cancer. Here, we show that the Vitamin D analog might be selective for transformed cells. The population at high risk of developing cancer is assumed to be initiated for carcinogenesis and, as we have shown, initiated cells respond well to $1\alpha(\text{OH})\text{D}_5$. In addition, we also showed here that $1\alpha(\text{OH})\text{D}_5$ is effective against steroid-responsive cancer cells. These results suggest that $1\alpha(\text{OH})\text{D}_5$ can be considered as a possible chemopreventive and therapeutic agent. Moreover, if given in combination with other agents, it may provide synergistic protection.

It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle

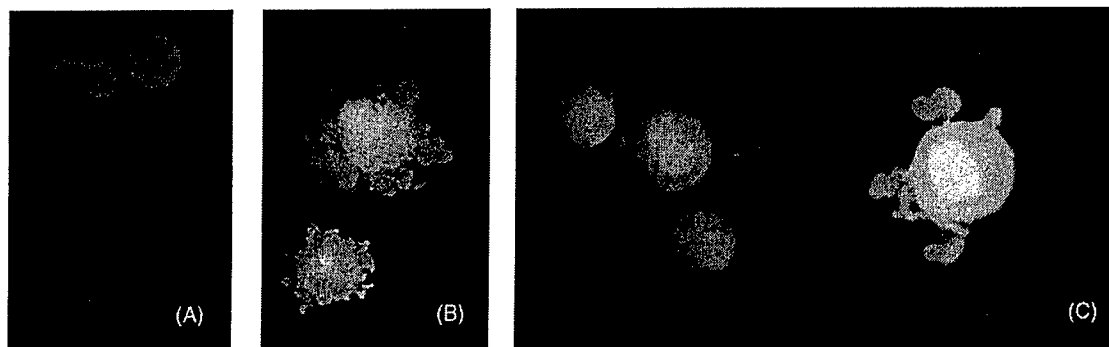


Fig. 8. Induction of apoptosis in BT474 cells by $1\ \mu\text{M}$ $1\alpha(\text{OH})\text{D}_5$, as determined by acridine orange and ethidium bromide staining. (A) control; (B) $1,25(\text{OH})_2\text{D}_3$ ($0.1\ \mu\text{M}$); (C) $1\alpha(\text{OH})\text{D}_5$ ($1\ \mu\text{M}$).

and prerequisite of chemoprevention is that the agent should not have any adverse effects. The lack of toxicity of $1\alpha(\text{OH})\text{D}_5$ at an effective concentration may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here the chemopreventive properties of a relatively new non-toxic analog of Vitamin D, $1\alpha(\text{OH})\text{D}_5$, against mammary carcinogenesis models. In addition, our results suggest that $1\alpha(\text{OH})\text{D}_5$ may be active selectively against transformed cells without showing adverse effects on normal breast epithelial cells.

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that Gleevec inhibits Rad51 activation and may potentially interfere with the repair of IR-induced DSB. Given the relationship between DSB repair and IR-induced cell killing, clonogenic survival analysis was performed on U251 glioma cells treated with a combination of Gleevec and radiation. Pretreatment of glioma cells with Gleevec resulted in a significant enhancement in the level of radiation-induced cell death. However, in contrast, Gleevec had no effect on radiation-induced cell killing of a non-immortalized normal human fibroblast cell line (C-2A). The effects of Gleevec on the radiosensitivity of tumor versus normal cells may be a result of the differential expression of Rad51. Immunoblot analysis showed that the glioma cells contained 4-6-fold higher levels of Rad51 than the normal cell line. Thus, these results indicate that Gleevec enhances radiation-induced tumor cell killing and suggest that the mechanism of Gleevec-mediated radiosensitization involves the inhibition of IR-induced Rad51 activity. Furthermore, Rad51 may be an appropriate target for selectively enhancing the radiosensitivity of tumor cells versus normal cells.

#966 Identification of a novel inhibitor of eukaryotic Elongation Factor-2 Kinase (calmodulin-dependent kinase III) as a potential anti-cancer drug. Arora, Jin-Ming Yang, Ryutaro Utsumi, Terry Kinzy, Tadashi Okamoto, Takashi Kurayama, and William Hait. *Cancer Institute of New Jersey, UMDNJ, New Brunswick, NJ, Kinki University, Nara, Japan, and University of Medicine and Dentistry, Piscataway, NJ.*

Protein translation represents a potentially important area for drug target discovery in cancer. Eukaryotic elongation factor-2 kinase (eEF-2K) phosphorylates and inactivates eukaryotic elongation factor-2 (eEF-2) causing a transient arrest in protein synthesis. Previous work from our laboratory demonstrated that the activity of eEF-2K is increased in several cancer cell lines and non-specific inhibition of this kinase decreased cell viability. Because the predicted secondary structure of eEF-2K resembled EnvZ, a bacterial histidine kinase, we screened a series of compounds known to inhibit bacterial histidine kinases. eEF-2K activity was measured using a recombinant His-tagged eEF-2 under assay conditions that ensured linearity with respect to amount of enzyme and time of incubation. Of 24 compounds representing three structural series, we identified eight derivatives with activity of $< 10 \mu\text{M}$. NH125 had an IC_{50} of 60 nM and was further evaluated as a lead compound. NH125 showed far less activity against protein kinase A ($\text{IC}_{50} = 80 \mu\text{M}$), protein kinase C ($\text{IC}_{50} = 7.5 \mu\text{M}$) or calmodulin-dependent kinase II ($\text{IC}_{50} > 100 \mu\text{M}$). Treatment of C6 glioma cells with NH125 for 12 hrs. decreased phosphorylation of cellular eEF-2 as measured with anti-phospho-eEF-2 antibody. The efficacy of NH125 against various cancer cell lines was determined by MTT assay. (Table 1). NH125 blocked cells at the G1/S boundary. In summary we identified a potent inhibitor of eEF-2K which demonstrated activity against cancer cell lines.

Table 1: NH 125 Inhibits the growth of cancer cell lines.

Cell Line	Origin	IC_{50} (μM)
C6 glioma	Glioma(rat)	1.0
U251 glioma	Glioma(human)	0.7
PC9 prostate	Prostate(human)	0.7
KB-3-1	Cervical(human)	1.5
KB-3-1	Oral(human)	0.9
OVCA9-3	Ovarian(human)	2.0

PREVENTION/SURVIVORSHIP RESEARCH 2: Natural Products Cancer Prevention

#967 Anti-angiogenic effect of grape seed extract in human prostate cancer xenograft in athymic mice. Chapla Agarwal, Sivanandhan Dhanalakshmi, Rana P. Shetty, and Rajesh Agarwal. *University of Colorado Health Sciences Center, Denver, CO.*

Tumor angiogenesis is an essential event in growth and metastatic spread of cancer. It has been suggested that preventive/therapeutic inhibition of angiogenesis might be exploited as a novel means of controlling cancer growth by natural or synthetic agents. Grape seed extract (GSE) is a natural rich source of bioflavonoids, and has shown several health-related benefits. Here, for the first time we provide evidence for the anti-angiogenic effects of grape seed extract on human umbilical endothelial cells (HUVEC) and human prostate tumor xenograft in athymic mice. Treatment of HUVEC cells with 25 and 50 $\mu\text{g}/\text{ml}$ doses of GSE for 24 h resulted in 71-91% ($P < 0.001$) cell growth inhibition and 30-43% ($P < 0.05$ - $P < 0.005$) cell death as compared to DMSO treated control (8.5% cell death). In MTT assay, similar GSE treatment for 24 h reduced HUVEC cell viability by 28-64% ($P < 0.022$ - $P < 0.001$). We also studied the effect of similar GSE treatment on cell proliferation by BrdU incorporation assay, which showed 65-74% ($P < 0.001$) inhibition in DNA synthesis. Further, nature of GSE-caused cell death in HUVEC was investigated by annexin V+propidium iodide staining and flow cytometric

analysis, which showed 17-43% ($P < 0.05$) apoptotic cell death at these doses of GSE after 24 h of treatment as compared to control with only 3% apoptotic cells. Next, we studied the HUVEC-associated MMP-2 activity by gelatin substrate gel zymography, in which 25-50 $\mu\text{g}/\text{ml}$ doses of GSE inhibited MMP-2/gelatinase A by 47-86%. We also observed that GSE strongly inhibits HUVEC capillary tube formation on Matrigel. In other studies, GSE (40-80 $\mu\text{g}/\text{ml}$ for 12 h) significantly inhibited (47-70%, $P < 0.01$) vascular endothelial growth factor (VEGF) secretion from DU145 cells in culture. In vivo DU145 tumor xenograft study, GSE treatment at 100 and 200 mg/kg body weight for 7 weeks (5 days/week by oral gavage) resulted in 64-75% ($P < 0.001$) decrease in CD31 (a specific marker of endothelial cell) positive cells in tumors as compared to saline-fed control tumors, which also correlated with tumor growth inhibition by GSE. These studies suggest that GSE has strong anti-proliferative and apoptotic effects on HUVEC accompanied with inhibition of MMP-2 and capillary tube formation. In vitro antiangiogenic potential of GSE is further substantiated by its in vivo inhibitory effect on tumor angiogenesis in advanced prostate carcinoma xenograft, thereby suggesting its role in angioprevention of prostate cancer.

#968 Efficacy of 1α -hydroxyvitamin D5 on colon carcinogenesis. Genoveva Murillo, Juliana K. Choi, and Rajendra G. Mehta. *University of Illinois at Chicago, Chicago, IL.*

The physiologically active form of vitamin D, $1\alpha,25$ -dihydroxyvitamin D3, plays an important role not only in the establishment and maintenance of calcium metabolism, but also in regulating cell growth and differentiation. The clinical usefulness of this natural metabolite of vitamin D is limited by its role in inducing hypercalcemia, and therefore, new analogs with a better therapeutic profile have been synthesized. Our laboratory synthesized an analog of vitamin D, 1α -hydroxyvitamin D5 [$1\alpha(\text{OH})\text{D5}$], and has shown its efficacy in several experimental models including breast. In the present study, $1\alpha(\text{OH})\text{D5}$ was evaluated for its chemopreventive activity on colon carcinogenesis. Since the formation of aberrant crypt foci (ACF) in response to carcinogen exposure is considered one of the earliest recognizable pre-cancerous lesions, the ACF model has been extensively used to identify modulators of colon carcinogenesis. We investigated the effects of $1\alpha(\text{OH})\text{D5}$ on multiplicity and size of azoxymethane (AOM)-induced ACF in mice. CF-1 mice were treated s.c. with 10 mg/kg AOM weekly for 2 weeks. $1\alpha(\text{OH})\text{D5}$ was supplemented in the diet (25 $\mu\text{g}/\text{kg}$ diet) beginning 7 days before the carcinogen treatment and was continued until termination of the study. Colons were stained with methylene blue and scored for the presence of ACF. $1\alpha(\text{OH})\text{D5}$ significantly ($p < 0.001$) reduced multiplicity of ACF from 29 ± 3.2 in the control to 1.4 ± 0.5 in the treatment group resulting in 95% suppression of ACF formation. Ongoing studies are being conducted to examine the effects of $1\alpha(\text{OH})\text{D5}$ during the initiation and post-initiation stages of colon carcinogenesis. The mechanism of action for $1\alpha(\text{OH})\text{D5}$ was investigated in several human colon cancer cell lines. Incubation of colon cancer cells with $1\alpha(\text{OH})\text{D5}$ ($0.1 \mu\text{M}$ to $2 \mu\text{M}$) for 3 days was found to inhibit cell growth in a dose dependent manner in 4 of the 5 cell lines studied. The cell cycle analyses indicated that the cells treated with $1\alpha(\text{OH})\text{D5}$ arrested in G1 by day 3 and remained in arrest until day 6. Immunocytochemistry studies showed an up-regulation of VDR in SW480 and HT-29 cells treated with $1\alpha(\text{OH})\text{D5}$, while no significant difference in VDR number was found in the SW620 (low VDR expressors). These results were confirmed by western blot analyses in HT-29 cells. Similarly, PPAR- β expression was shown to be up-regulated by 2-fold in cells treated with $1 \mu\text{M}$ $1\alpha(\text{OH})\text{D5}$. Western blot analyses also showed that treatment of $1\alpha(\text{OH})\text{D5}$ resulted in down-regulation of E2F1 and Cyclin B expression following 3 and 5 days of treatment with $1\alpha(\text{OH})\text{D5}$, respectively. These results lend support to the potential use of $1\alpha(\text{OH})\text{D5}$ as a candidate for chemoprevention and treatment of colon cancer. (Supported in part by Functional Foods for Health Program, University of Illinois).

#969 Chemopreventive effects of curcumin on B[a]P-induced carcinogenesis in the hamster cheek pouch model. Jimi L. Brandon, Miriam A. Rogel, Claudio J. Conti, John DiGiovanni, Robert A. Newman, and Irma B. Gimenez-Conti. *University of Texas MD Anderson Cancer Center, Smithville, TX and University of Texas MD Anderson Cancer Center, Houston, TX.*

The present study was carried out to examine the chemopreventive effects of curcumin on the hamster cheek pouch carcinogenesis model. This model of oral carcinogenesis has been widely used in chemoprevention studies. Curcumin is a known antioxidant and anti-inflammatory agent, and it has been found to reduce carcinogenesis in various animal models including mouse skin, forestomach, intestine, lung, and rat breast. Benzo[a]pyrene (B[a]P) is a ubiquitously distributed environmental carcinogen. Using B[a]P and a complete carcinogenesis protocol we have been able to produce pre-cancerous (leukoplakia-like lesions) at 16 weeks of treatment and squamous cell carcinomas at 20 weeks of treatment. These lesions are primarily endophytic and resemble those that develop in human oral mucosa.



EFFECT OF VITAMIN D ANALOG (1 α HYDROXY D5) IMMUNOCONJUGATED TO Her-2 ANTIBODY ON BREAST CANCER

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We previously showed that a new vitamin D analog, 1 α (OH)D5 (D5), induced differentiation and inhibited the growth of breast cancer cells. In this report, we examined whether D5 specifically delivered to breast cancer cells could have any therapeutic effect. D5 was linked to Her-2 antibody using sulfosuccinimidyl 6-4 azido nitrophenylamido hexanode (SANPAH) as a linker. The Her-2 antibody selected in our study had no significant effect on the *in vitro* or *in vivo* growth of breast cancer cells; however, it had cell-differentiating action. *In vitro*, D5-Her-2 antibody conjugate (IMC) showed the ability to specifically bind to Her-2-expressing cells, to compete with Her-2 antibody for surface receptor and to cause internalization. IMC (equivalent to 5 μ g Her-2 antibody given intraperitoneally once weekly for 6 weeks) significantly inhibited the growth of BT-474 cells transplanted into athymic mice. The *in vivo* growth-inhibitory effect of IMC treatment was similar to that observed in animals receiving D5 continuously as a dietary supplement. These results show that the targeted delivery of D5 by immunoconjugation to cell surface receptor antibodies may be of potential therapeutic value for the treatment of Her-2 positive breast cancer.

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Key words: vitamin D; analog; immunoconjugate; Her-2

Even though recent introduction of breast screening mammography and highly sensitive diagnostic procedures has helped in the early diagnosis of breast cancer, control of metastatic disease remains a major concern of oncologists.¹ Many breast cancers, especially those showing overexpression of Her-2 protooncogene, are more aggressive and are characterized by early metastatic spread and reduced patient survival.^{2–6} Importantly, overexpression of Her-2 protein is associated with altered clinical responsiveness to some standard chemotherapy regimens.^{2,7,8} Thus, new therapeutic approaches are urgently needed for treating highly aggressive Her-2-overexpressing breast cancers. Special emphasis has been given to the search for the agents that have both antiproliferative and cell-differentiating actions on breast cancer cells. Vitamins, especially the vitamin D metabolite 1,25(OH)D3, have been shown to (i) suppress the development and progression of breast cancer and other carcinomas *in vivo* and *in vitro*; (ii) inhibit the metastatic spread of tumor cells and (iii) promote differentiation of breast cancer cells.^{9–16} However, the concentration necessary for the growth inhibitory effect on cancer cells *in vivo* causes hypercalcemia.^{17,18} Thus, the recent focus is to identify highly effective but nontoxic vitamin D derivatives.

We showed earlier that a novel vitamin D analog (D5) has potent cell-differentiating and antiproliferative actions in breast cancer cells.^{15,16} *In vitro*, after 7 days exposure of breast cancer cells to 0.1–1 μ M D5, we observed the induction of various biomarkers associated with cell differentiation, such as an increase in intracellular accumulation of neutral lipid, an increase in intracellular casein and the enhanced expression of α 2 integrin.^{15,16} Human breast cancer cells exposed *in vitro* to D5 also lost their tumorigenic potential when transplanted into athymic mice.¹⁵

Interestingly, D5 supplemented in the diet inhibited *in vivo* growth of breast cancer cells in athymic mice.¹⁵ D5 also reduced the incidence of carcinogen-induced mammary tumors in the experimental rat model.¹⁹ In our study, we examined the possible therapeutic potential of targeted delivery of D5 to Her-2-overex-

pressing breast cancer cells by using an immunoconjugate linked to the Her-2 antibody in an experimental model.

MATERIAL AND METHODS

Human breast carcinoma cell lines

The breast carcinoma cell line BT-474 was obtained from the American Type Culture Association (ATCC; Rockville, MD). The cells were maintained in MEM-E (minimum essential medium containing Earl's salt) supplemented with 10% FBS, essential amino acids, glutamine and streptavidin fungizone.

Vitamin D analog and the Her-2 antibody

D5 was purchased from Onquest, Inc. (Chicago, IL). For both *in vitro* and *in vivo* studies, D5 was originally dissolved in 100% ethanol. For *in vitro* studies, stock solution was diluted and then added to the culture medium so that the final concentration of ethanol in the medium was less than 0.01%. Antibody (BSA-free, azide-free) against the extracellular domain of Her-2 receptor used for both *in vivo* and *in vitro* studies was obtained from Neomarkers (Fremont, CA). The Her-2 antibody (clone 9G6.10, ab-2) is reported to immunoprecipitate 160 kDa protein from extracts of Her-2-positive cells.²⁰ The antibody has no growth-inhibitory action *in vivo* in the breast cancer xenograft model. Control immunoglobulin (Ig)G isotype matched antibody (mouse IgG, clone NC G01, BSA-free, azide-free) was obtained from Neomarkers (Fremont, CA).

Preparation of the D5-Her-2 antibody immunoconjugate

D5 was covalently linked to antibodies (Her-2 antibody or control antibody) using sulfosuccinimidyl 6-4 azido nitrophenylamido hexanode (SANPAH; Pierce, Rockford, IL) as a linker. Immunolinking of D5 was performed by the 2-stage method based on photoaffinity crosslinking. In brief, the crosslinking agent SANPAH was first coupled to the amino group of the antibody. The antibody (1 mg/ml = 6.67 μ M final concentration) was incubated in the dark with different molar concentrations of SANPAH as a linker (133.4–533.6 μ M final concentration). After incubation, unreacted SANPAH and hydrolyzed linker by-product were removed by G-25 sephadex gel filtration. Different molar concentration of D5 (133.4–533.6 μ M final concentration) was added to the reaction mixture and subjected to photo activation by 3–4 camera flash light exposures. The nitro-substituted aryl-azide,

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when photolysed, forms an aryl nitrene that reacts nonselectively with D5 to form a covalent bond. Excess vitamin D analog was removed by dialysis. Unconjugated antibody was not removed from the reaction mixture. Each preparation was tested for the specific binding ability to Her-2 receptor on BT-474 cells. The immunoconjugate (IMC) preparation was loaded on a 4–20% SDS polyacrylamide gel. After being electrophoresed, the gel was exposed overnight to Kodak radio autography film in the dark at 4°C. If nonradioactive antibody was used in the IMC preparation, the SDS-PAGE gel was immunoblotted on immunoblon paper, subjected to secondary anti-mouse IgG conjugated to horseradish peroxidase (HRP) and then subjected to chemiluminescence analysis using a kit from Amersham Pharmacia Biotech (Piscataway, NJ). All batch preparations were tested for electrophoretic mobility before use in the experiments.

Competitive binding of IMC with the Her-2 antibody

BT-474 cells were seeded at 20,000 cells/well density in 96-well plates. Cells were allowed to attach to the culture wells overnight. Cells were washed twice with PBS and were fixed in 0.06% glutaraldehyde. After washing with PBS, cells were incubated for 10 min in PBS containing 10% FBS to reduce the nonspecific binding. Cells were then incubated with 125 I-labeled Her-2 antibody (S.A. = 1.8 μ Ci/ μ g, 10 ng) or 125 I IMC (S.A. = 1.2 μ Ci/ μ g, 10 ng) alone or in the presence of increasing concentrations (0–750 ng) of unlabeled Her-2 antibody. 125 I antibody was aliquoted in the wells without cells and was processed along with samples as a blank. We also incubated the 125 I-labeled Her-2 antibody with increasing concentrations of the control antibody or with its IMC with D5. The cells were incubated at RT for 2 h and then washed with PBS twice. Radioactivity was extracted by incubating the cells in PBS containing 1% SDS at room temperature for 15 min. Liquid was absorbed from the wells using cotton swabs, and the radioactivity in the cotton swabs was counted using a gamma counter. Radioactivity detected in the blank wells was considered as nonspecific counts and was subtracted from binding detected to the cells in the presence/absence of unlabeled antibody. Data represent percent binding in the absence of unlabeled antibody. Each data point is a mean value \pm standard error (SE) of 3 independent observations.

Internalization of IMC

For the internalization assay, BT-474 cells growing on sterile glass cover slips were incubated with 1.5 μ g/ml Her-2 antibody or control antibody alone (in medium containing 5% charcoal-stripped FBS) or IMC (D5 linked to control or Her-2 antibody, equivalent to 1.5 μ g/ml antibody) at 37°C for 0–24 h. At the end of incubation, cells were washed extensively with PBS, fixed in 10% buffered formalin and then permeabilized in cold methanol, washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Dako Corp., Carpinteria, CA) for 30 min. After washing, cells were coverslipped using vectashield aqueous mounting media containing DAPI (4, 6-diamidino-2-phenylindol, 1.5 μ g/ml; Vector Laboratories, Inc., Burlingame, CA). Fluorescence images were obtained using a Carl Zeiss LSM 510 laser scanning confocal microscope (BioRad, Richmond, CA) equipped with a X63 water emulsion objective. Beams of 488 nm and 364 laser were used for excitation and blue (DAPI) and green (FITC) fluorescence were recorded through LP505 and LP470 filters, respectively. For quantitative analysis, cells growing in a monolayer were incubated for 0–24 hr in the presence of the Her-2 antibody and IMC, washed with PBS, fixed in 0.5% buffered formalin and then treated with ice cold methanol for 5 min. After PBS wash, the cells were incubated with FITC-labeled secondary antibody for 30 min and then washed with PBS. Relative fluorescence intensity was determined by fluorescence activated cell sorting (FACS) analysis (Epic Elite Flow Cytometer, Coulter Corporation, Miami, FL). Data are presented as fluorescence intensity recorded in arbitrary units.

Immunostaining for Ki-67 and intracytoplasmic casein

To determine the *in vitro* effect on cell proliferation, Ki-67 immunostaining was used as a biomarker. For differentiation, accumulation of casein granules was determined immunohistochemically in the cells. BT-474 cells were plated on Nunc culture cover slips and allowed to attach to the culture wells overnight in MEM-E containing 10% FBS. The next day, the culture medium was replaced with SS medium (MEM-E containing 5% charcoal-stripped FBS) containing ethanol as a vehicle, 1 μ M D5 (428 ng/ml), Her-2 antibody (1.5 μ g/ml), control antibody (1.5 μ g/ml), or IMC of respective antibodies (1.5 μ g Her-2 antibody/control antibody containing 171.2 ng of D5 assuming that all D5 used in IMC preparation gets immunoconjugated). The cells were incubated at 37°C in the atmosphere of 95% air and 5% CO₂. Medium was changed with respective ingredients on day 4 after initiation of the treatment. The cells were thoroughly rinsed in PBS and fixed in 10% buffered formalin followed by ice-cold methanol. The cells were first incubated with anti-mouse FITC-labeled antibody to avoid interference of Her-2 antibody/immunoconjugate preincubation in Ki-67 staining. Ki-67 staining was performed in fixed cells by the method described previously.^{15,16} Cells were counterstained using hematoxylin. Only AEC (3-amino-9-ethylcarbazole; Biogenex, San Ramon, CA) staining in the nucleus was considered positive; the number of positive cells/total number of cells was counted in 10 different high-power fields, and the percentage of cells positive for Ki-67 were calculated. The experiments were repeated using 10 different cover slips in each treatment group, and the data represent percent mean \pm SE in a minimum of 10 different cover slips.

For casein staining, the cells were incubated with rhodamine-labeled anti-mouse secondary antibody to prevent the interference from the Her-2 antibody preincubation in casein immunostaining. The cells were washed thoroughly with PBS, incubated with anti-casein antibody (Neomarker, Fremont, CA; 1:100 dilution), incubated with FITC-labeled secondary antibody (Dako, Carpinteria, CA) and visualized under a microscope.

Lipid staining in BT-474 cells

The cells cultured on the cover slip were treated as described above. After incubation, the cells were immediately washed with Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, 1M)

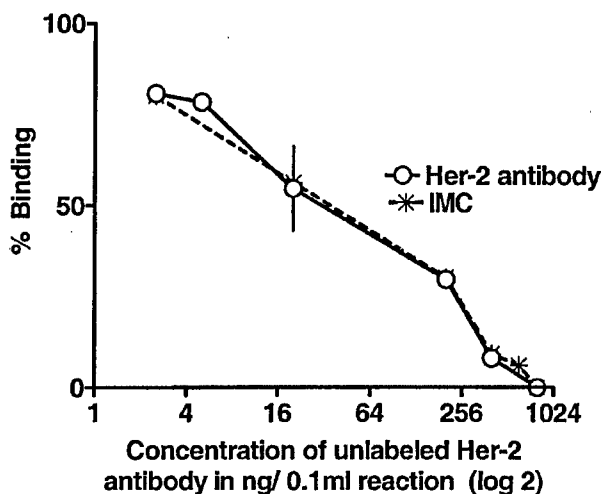


FIGURE 1 – BT-474 cells (20,000 cells/well) were incubated for 2 h at room temperature with 125 I-labeled Her-2 antibody or 125 I Her-2 1 α (OH)D5 IMC alone or in the presence of increasing concentrations of unlabeled Her-2 antibody. After PBS wash, radioactivity was extracted and counted using a gamma counter. Each data point represents the mean \pm SE of 3 independent observations.

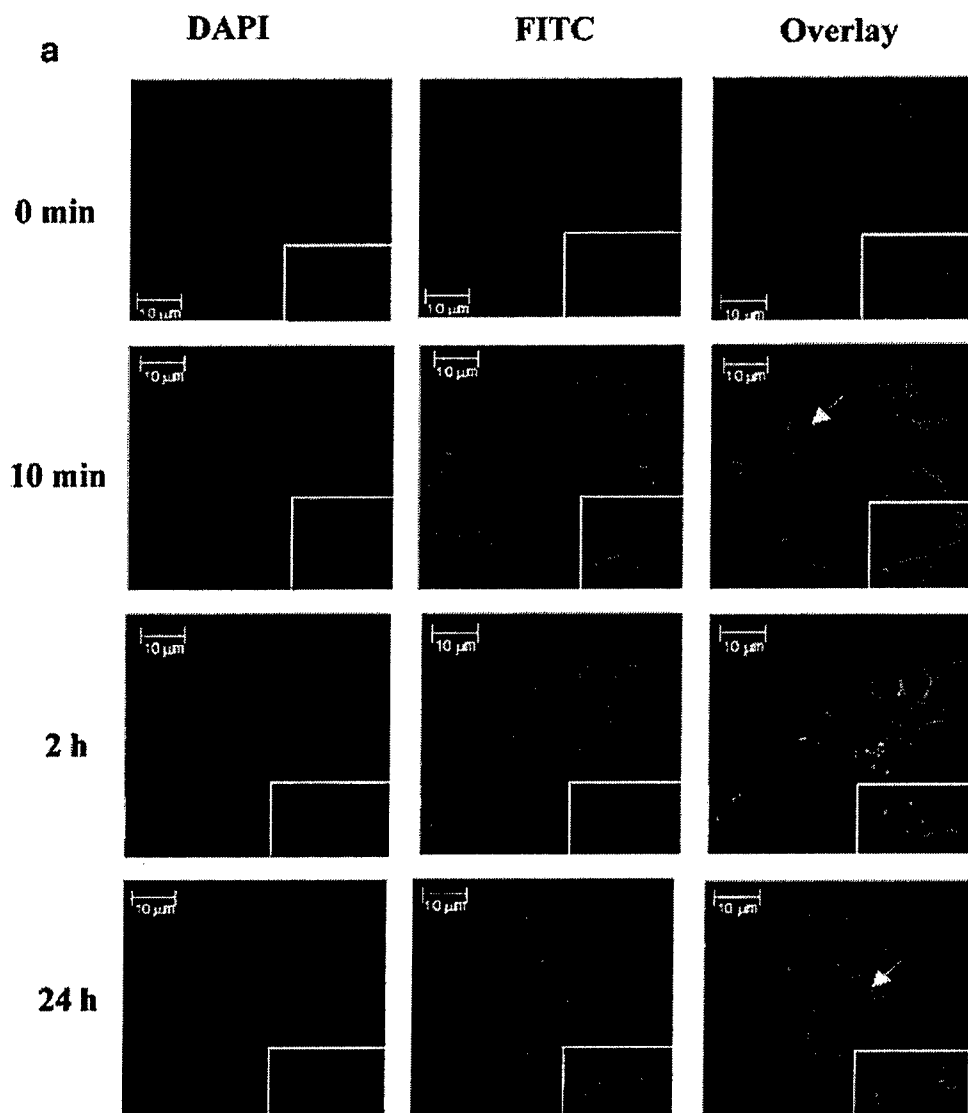


FIGURE 2 – BT-474 cells were incubated with (1.5 µg/ml) Her-2 antibody (a) or IMC (b) at 37°C for 0–24 h. At the end of incubation, cells were washed, fixed in 10% buffered formalin and permeabilised in cold methanol, then incubated with FITC-conjugated anti-mouse IgG. The cells were counterstained with DAPI and the images were taken using a confocal laser scanning microscope. Insets represent cropped images of the isolated cells. Arrow shows cell surface (10 min) or intracellular vesicular (24 h) immunostaining. Quantitative analysis of Her-2 antibody and IMC in BT-474 cells (c). Cells were incubated at 37°C with 1.5 µg/ml Her-2 antibody or IMC for 0–24 h. Accumulation of Her-2 antibody or IMC in the cells was determined by incubating cells with FITC-labeled anti-mouse IgG. Relative fluorescence intensity was determined using a flow cytometer. The bar graph shows representative data of 3 independent experiments. Data represent mean \pm SE.

buffer and then stained with Nile Red (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in acetone, diluted to 1:200 in Hepes buffer) stain for 5 min. The cells were immediately visualized under fluorescence microscope (using a blue filter) and photographed. To further confirm that the staining observed was due to neutral lipids, a set of coverslips was rinsed in isopropyl alcohol before staining with Nile Red stain.

The effect of Her-2 antibody, D5 and IMC on in vivo growth in breast cancer cells

Animals were obtained from Frederick Cancer Research Facility (Bethesda, MD). BT-474 cells (1 million cells/animal) were suspended in a mixture (1:1 vol./vol.) of HBSS (Hank's balanced salt solution; Invitrogen Corporation, Carlsbad, CA) and Matrigel (BD Sciences, Bedford, MA), then injected subcutaneously into the

dorsal flank region of 3- to 4-week-old female Balb/c athymic mice. All animals received a subcutaneous estrogen pellet (0.72 mg/animal, 60 days release; Innovative Research, Saratoga, FL). Once the palpable tumor developed (0.03 cm³), animals were divided into various groups: (i) receiving regular powdered diet mixed with ethanol as vehicle (control diet); (ii) receiving D5 (12.5 µg/kg diet)-supplemented powdered diet; (iii) receiving intraperitoneal injection of Her-2 antibody (5 µg/animal, once weekly) and regular powdered mouse diet as in the first group; (iv) receiving IMC (equivalent to 5 µg Her-2 antibody) once weekly intraperitoneally and receiving the control diet. Because we did not observe any effect of control antibody and control antibody IMC on *in vitro* growth of BT-474 cells, these treatments were not further tested in *in vivo* study. D5 contents in various *in vivo* administrations are

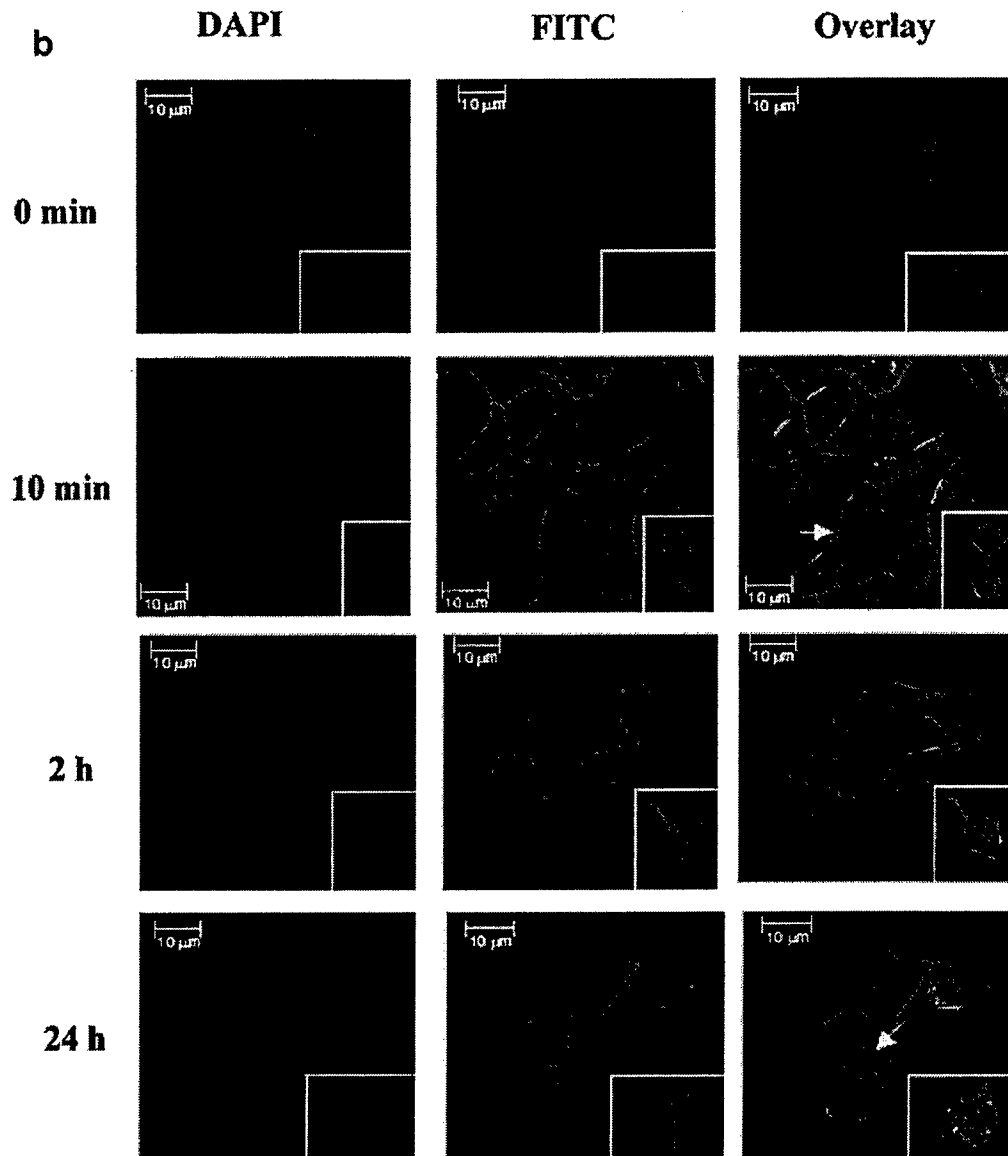


FIGURE 2 – CONTINUED.

estimated as follows. Dietary D5 was given at 12.5 $\mu\text{g}/\text{kg}$ dose. On average, diet consumption by an athymic mouse is between 6–7 g/day. Thus, the amount of D5 consumed by an animal each day ranges between 75–87 ng/day or 525–621 ng/week. For IMC treatment, 5 μg IMC contains (assuming 100% of D5 added to 1 ml of antibody gets immunolinked) 570.9 ng D5. IMC was further diluted with HBSS to get a final injection dose of 5 μg IMC/100 μl HBSS. In a separate experiment, we also tested whether intraperitoneal administration of D5 (D5 stock solution was made to obtain 266.8 μM final concentration and then further diluted to give 570.9 ng/ml D5 for injection into animals) given once weekly for 4 weeks has any effect on the growth of BT-474 xenograft.

Both the control and D5-supplemented diets were given to the animals in sterile food cups; an equal amount of food was placed in each cup. Food cups were protected from direct light exposure. Food cups were changed twice weekly. For preparation of D5-supplemented diet, a known amount of D5 was dissolved in absolute ethanol and then mixed with powdered mouse chow

(Teklad, Madison, WI) using a diet mixer. Diet was stored in foiled containers to protect from light, and stored at 4°C. The stability of D5 was determined periodically.

An aliquot of the diet was extracted with methanol, and the extract was subjected to high performance liquid chromatography (HPLC) analysis. The control diet was mixed with ethanol (equal to that used for D5 diet) only. Ethanol from the diet mixtures was evaporated by placing it at room temperature for 20 min in sterile culture hoods. All animals received water *ad libitum*. Each group consisted of a minimum of 5 animals. The experiment was repeated twice. The animals were examined once weekly for growth of tumor at the site of injection, and tumor size was monitored using calipers. Tumor volume was calculated as cm^3 using the following formula: tumor volume (cm^3) = $3.14/6 \times \text{length} \times \text{width} \times \text{depth}$. Data represent the mean tumor volume \pm SE (cm^3) in each group. The animals were sacrificed at the indicated time unless they appeared to be moribund or tumors showed sign of necrosis. At termination, blood was collected for calcium determination.

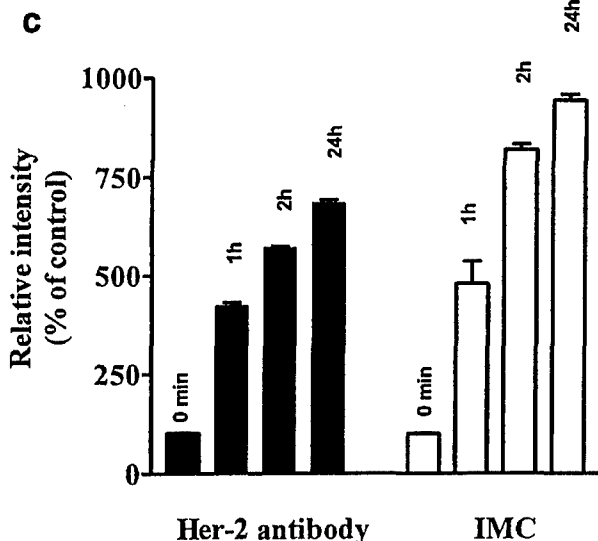


FIGURE 2 - CONTINUED.

Determination of serum calcium levels

Quantitative determination of serum calcium levels was performed by colorimetric analysis using calcium (Arsenazo III) reagent (Sigma Diagnostics, St. Louis, MO). Calcium in the serum reacts to the Arsenazo III reagent and forms purple-colored calcium-Arsenazo III complex. The intensity of the color is read at 600 nm and evaluated in relation to the color intensity obtained by processing simultaneously known calcium standards (Sigma Diagnostics, St. Louis, MO). Data represent the mean \pm SE values obtained in 5 animals.

Statistical analysis

Statistical significance between groups was determined by ANOVA. Differences between group mean values were determined using Dunnett's or Bonferroni multiple comparison tests. A p -value < 0.05 was considered a statistically significant difference. Data represent the mean \pm SE.

RESULTS

Characteristics of IMC

The D5-Her-2 IMC prepared with different molar concentrations of antibody to SANPAH was subjected to a 4–20% SDS-PAGE. After electrophoresis, the molecular weight of the IMC was increased with higher molar concentrations of antibody to D5. At higher molar concentrations of SANPAH, electrophoretic mobility of the antibody SANPAH was decreased. We used SANPAH at 1:40 concentration and prepared IMC with different molar concentrations of D5. However, at 1:40 antibody to SANPAH and 1:40 antibody to D5 molar concentration (6.67 μ M antibody: 266.8 μ M of SANPAH and D5 each), the IMC retained the ability to bind to Her-2 receptors. Thus, in all experiments, the IMC was prepared using 1:40:40 molar (Her-2 antibody:SANPAH:D5) concentration.

Competitive binding of IMC with Her-2 antibody for Her-2 receptor binding sites in BT-474 cells

As shown in Figure 1, the percent binding of both the 125 I Her-2 antibody and the 125 I IMC was reduced when the cells were incubated with increasing concentrations of the unlabeled antibody, suggesting that both the 125 I Her-2 antibody and the 125 I Her-2 antibody immunolinked to D5 compete with the unlabeled Her-2 antibody for Her-2 receptor binding sites in BT-474 cells

(Fig. 1). The nonradioactive control antibody or its IMC failed to compete with the 125 I Her-2 antibody (data not shown).

Internalization of the IMC in BT-474 cells

We examined whether the Her-2 antibody, the isotype matched control nonspecific antibody or their IMC linked to D5 are internalized in BT-474 cells showing over expression of Her-2 receptor. At 0 min time (untreated cells), we did not observe any FITC signal in the cells. After 10 min incubation with the Her-2 antibody, intense immunostaining was observed on the cell surface; after 30–60 min incubation, immunostaining was mostly on the cell surface but a few vesicular bodies were also detected in the cytoplasm. After 2 h incubation, the cell surface staining was present but cytoplasmic vesicular staining had increased. At 24 h, numerous FITC-labeled vesicular bodies were detected in the cytoplasm. Figure 2a shows representative confocal images of DAPI-stained cells, FITC-labeled cells and combined pictures showing DAPI and FITC labeling obtained in BT-474 cells after 0, 10 min, 2 hr and 24 hr incubation with the Her-2 antibody. Insets in each image show cropped images of isolated cells.

We also determined the internalization of IMC. BT-474 cells were incubated with IMC for 10 min to 24 h, and then the cells were incubated with FITC-labeled secondary antibody and the localization of IMC (Her-2 covalently linked to D5) was determined under a laser scanning confocal microscope. In general, the immunostaining pattern was similar to that observed with the Her-2 antibody. In untreated (0 min) cells, no immunostaining was detected in BT-474 cells. Distinct cell surface immunostaining was observed between 10 min to 2 h. We also observed the presence of vesicular bodies in the cytoplasm after 2 h, and this was at maximum level at 24 h (Fig. 2b).

As experimental controls, we also simultaneously processed cells incubated with the control antibody or the IMC of control antibody. We did not observe cell surface or specific intracellular immunostaining at any time point during the course of our study (data not shown).

Quantitative analysis of time dependent accumulation of the Her-2 antibody or its IMC in BT-474 cells was performed by flow cytometry. Both Her-2 antibody and IMC showed a time dependent increase in fluorescence intensity; maximum intensity was noticed after a 24 h incubation with respective agents. Comparatively, fluorescence intensities were similar in cells incubated with Her-2 antibody and its IMC throughout the course of our study (Fig. 2c).

Effect of IMC on intracellular lipid accumulation in BT-474 cells

We examined the accumulation of intracellular lipid droplets as a marker of breast cell differentiation in BT-474 cells treated *in vitro* with D5, Her-2 antibody or IMC for 7 days. In all treatment groups, the cell surface was stained bright orange, whereas intracytoplasmic lipid droplets stained bright yellow. Lipid droplets were generally localized in the cytoplasm around the nuclear periphery. In vehicle-treated control cells, few small lipid droplets were noticed. On the other hand, compared to vehicle-treated control cells, we observed increased accumulation of bright yellow lipid droplets localized around the nuclear periphery in cells treated with Her-2 antibody, D5 or IMC (Fig. 3). Control antibody or IMC to control antibody showed few lipid droplets, similar to that in vehicle treated control (see inset in Fig. 3a and 3d) cells. In cells pretreated with isopropyl alcohol before Nile Red staining, lipid droplets were not observed, whereas cell surface orange staining was similar to that observed in cells stained without isopropyl alcohol treatment (data not shown).

Effect of IMC on casein accumulation in BT-474 cells

We examined accumulation of intracellular casein granules as a marker of breast cancer cell differentiation after various treatments. In control vehicle-treated cells, we did not observe casein granules in the cytoplasm. After treatment with D5, we observed

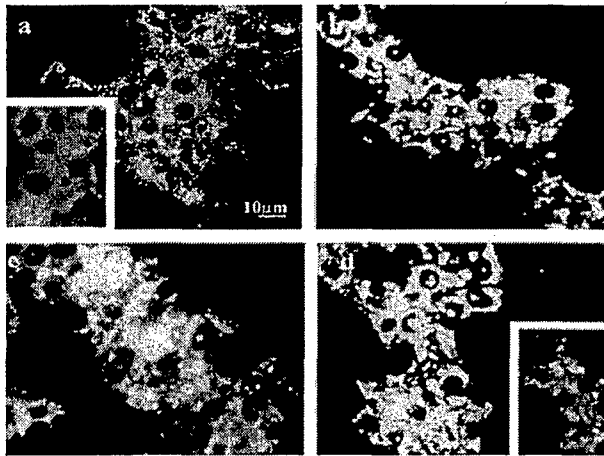


FIGURE 3—Lipid staining in BT-474 cells incubated for 7 days in MEM-E containing 5% charcoal-treated FBS alone (a) or containing 5 μ g/ml Her-2 antibody (b), 10^{-6} M $1\alpha(\text{OH})\text{D}_5$ (c) or IMC (d). At the end of incubation, cells were washed with HEPES buffer and then stained with Nile Red stain. Bright yellow stain indicates neutral lipid, and bright orange stain is due to phospholipid staining. Insets: treated with control antibody (a) or control antibody IMC (d).

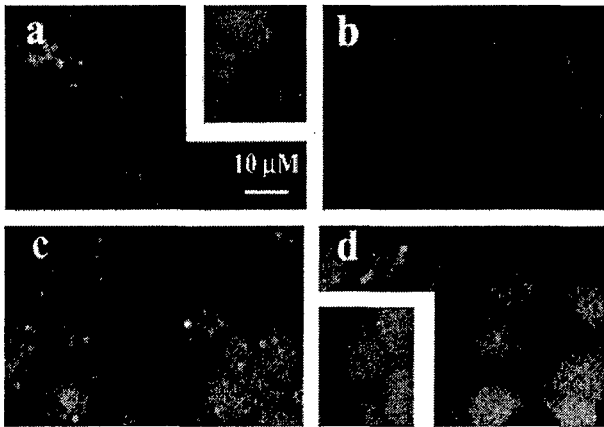


FIGURE 4—Beta casein immunostaining in BT-474 cells incubated in culture medium containing vehicle only (a), Her-2 antibody (1.5 μ g/ml) (b), 1 μ M $1\alpha(\text{OH})\text{D}_5$ (c) or IMC (equivalent to 1.5 μ g Her-2) (d). Green fluorescent granules show casein staining. Insets: treated with control antibody (a) or control antibody IMC (d).

presence of casein granules in the cytoplasm. Similarly, both Her-2 antibody- and IMC-treated cells exhibited more accumulation of casein granules than did vehicle-treated control cells. The immunostaining pattern in IMC-treated cells was similar to that observed in D5-treated cells. In both treatments, the accumulation of large distinct granules scattered in the cytoplasm was observed. Staining in antibody-treated cells and in control antibody IMC-treated cells was similar to that observed in vehicle-treated control cells (see inset in Fig. 4a and 4d).

Effect of IMC on *in vitro* proliferation of BT-474 cells

For proliferation, Ki-67 was determined in cells incubated for 7 days with various treatment agents or a combination of Her-2 antibody and D5. Ki-67 staining (%) was similar in cells treated with vehicle, Her-2 antibody, control antibody or control antibody IMC. BT-474 cells treated with D5 and IMC exhibited significant

($p < 0.05$) reduction in % Ki-67 stained cells. These results are presented in Table I.

Effect of IMC on *in vivo* growth of BT-474 cells in athymic mice

The effect of $1\alpha(\text{OH})\text{D}_5$ supplemented in the diet was examined in female athymic mice transplanted with BT-474 cells. In animals treated with dietary D5 supplement, tumor volumes (cm^3) were significantly ($p < 0.05$) smaller than those in the control group at days 21, 28 and 47 after initiation of the treatment. Mean tumor volumes in control and Her-2 antibody treated groups were similar throughout the course of our study. In animals treated with IMC, mean tumor volumes were significantly ($p < 0.05$) smaller than in the controls or HER-2 antibody-treated group, and were similar to that in the D5-treated group at 21–47 days after initiation of treatment (Fig. 5). In a separate experiment, we also determined the effect of D5 given intraperitoneally ($n = 8$) once weekly for 4 weeks. The control group ($n = 10$) received ethanol (solvent volume similar to that in D5 preparation). Mean tumor volume in animals given intraperitoneal D5 (data not shown in Fig. 5) was similar to that in the control group (day 23, 0.13 ± 0.02 vs. 0.13 ± 0.02 ; day 33, 0.30 ± 0.04 vs. 0.38 ± 0.05).

Serum calcium levels in animals treated with IMC

Serum calcium levels were slightly but not significantly higher in those animals treated with D5 than in vehicle-treated controls (9.37 ± 0.26 vs. 8.41 ± 0.39 mg/dl, $n = 5$ in each group). The Her-2 antibody treatment had no significant effect on calcium levels (7.99 ± 0.29 mg/dl, $n = 5$ in each group). In animals treated with IMC, serum calcium levels were significantly ($p = 0.05$) lower (7.45 ± 0.63 mg/dl, $n = 5$) than in those animals treated with the D5 in the diet; they were similar to the levels in the Her-2 antibody-treated group and were not significantly different from those in the control group.

DISCUSSION

We recently identified a vitamin D analog that induces differentiation-associated biomarkers in breast cancer cells; *in vivo* and *in vitro*, it inhibits growth of the selected human breast cancer cell

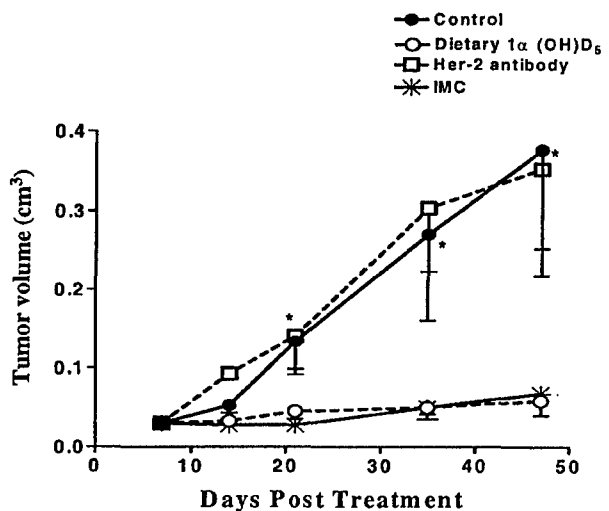


FIGURE 5—Mean tumor volume in control vehicle-treated animals receiving dietary supplement of $1\alpha(\text{OH})\text{D}_5$ (12.5 μ g/kg diet), animals treated with the Her-2 antibody (5 μ g) intraperitoneally once weekly during the course of study and animals treated with IMC intraperitoneally once weekly during the course of study. Data represent the mean tumor volume \pm SE in each group. *Indicates significant difference between control and D5 (dietary) and IMC treatment groups.

TABLE I—KI-67 IMMUNOSTAINING IN BT-474 CELLS¹

Treatment	% of cells stained
Control (vehicle only)	43.6 ± 5.7 (n = 10)
1α (OH)D5 (1 μM)	10.23 ± 2.3 (n = 10) ¹
Her-2 antibody (1.5 μg/ml)	45.18 ± 3.8 (n = 10)
Control antibody (1.5 μg/ml)	46.9 ± 2.89 (n = 10)
IMC	11.5 ± 6.7 (n = 13) ^{1,2,3}
Control antibody immunoconjugate	42.71 ± 1.67 (n = 10)

¹The cells were incubated at 37°C in the culture medium containing 5% FBS for 7 days in the presence of vehicle, D5, Her-2 antibody, IMC, control antibody or its immunoconjugate. Number in parentheses indicates number of independent observations. — ²Significant difference between control and treatment groups. — ³Significant difference between Her-2 antibody and IMC treated group. — ⁴Significant difference between IMC and control antibody immunoconjugate.

lines.^{15,16} Detailed preclinical toxicity studies suggested that this vitamin D analog is well tolerated in experimental animals.¹⁹ Although D5 is shown to be nontoxic in 28-day toxicity studies, if used continuously for a prolonged time period it could potentially develop a calcemic effect. Also, if the compound is linked to the cancer cell-specific receptor protein, it could be delivered to specific sites without affecting other normal tissues. In our study, we evaluated whether targeted delivery of D5 by linking it to a commercially available mouse monoclonal Her-2 antibody is effective in inhibiting the growth of breast cancer cells expressing high amounts of Her-2 protein. Even though it would be ideal to use herceptin (a humanized Her-2 antibody currently in use in the clinic), we used mouse monoclonal antibody, which by itself has no significant effect on the growth of breast tumor cells as a carrier protein. The latter characteristic allowed us to determine precisely whether targeted delivery of D5 to breast cancer cells has therapeutic benefit.

We confirmed that the Her-2 antibody used in our present study, when immunolinked to D5, retains its original properties and competes with intact unconjugated native antibody for the Her-2 receptor binding sites on breast cancer cells. Detailed time-course study also suggested that IMC undergoes internalization. Detailed time course analysis by confocal microscopy showed that both Her-2 antibody and IMC bind to the cell surface and then localize to the cytosol in vesicular form. Whether D5 linked to the Her-2 antibody dissociated from the IMC and then entered the nucleus is unclear. However, based on the effect of IMC on cell proliferation *in vivo* and *in vitro*, we speculate that once the IMC is internalized, the Her-2 antibody is degraded and then D5 is released in the cytosol and translocated to the nucleus.

We further confirmed that both D5 and the Her-2 antibody in the IMC retained their cell-differentiating properties. IMC is able to induce cell differentiation, as evident from the increased accumulation of intracellular lipid droplets. In our previous study, we showed that in MCF-7 and BT-474 cells, intracellular lipid accumulation is increased after D5 treatment compared to the vehicle-treated control.^{15,16} We confirmed the cell-differentiating action of IMC by examining another breast cell differentiation marker: beta casein. Generally, beta casein, a major component of milk, is produced by differentiated breast cells; breast cancer cells fail to produce casein. In our study, we observed the accumulation of casein granules in BT-474 cells treated with D5, the Her-2 antibody and IMC. These results collectively indicate that D5 and the Her-2 antibody individually have cell-differentiating action on BT-474 cells, and that the latter characteristic is preserved when these 2 compounds are immunoconjugated.

In vitro, D5 showed growth-inhibitory action in BT-474 cells, as evident from the percentage of Ki-67 positive cells after 7 days treatment. Similarly, IMC treatment also exhibited growth-inhibitory action. In contrast to cell-differentiating action, Her-2 antibody did not show an effect on cell proliferation. These results are in agreement with those reported previously by Van Leeuwen and associates.²⁰ In that report, the effect of 9G6.10 antibody was

determined in various cell types harboring normal or mutated Her-2 protein. The antibody 9G6.10 (40 μg/culture dish) showed no significant influence on colony formation in soft agar of those cells with normal Her-2 protein; however, it significantly inhibited the incidence of colony formation of cells transfected with mutant Her-2. BT-474 cells express the nonmutated Her-2 protein, and in our study the 9G6.10 Her-2 antibody had no effect on cell growth. Our results on the effects of the Her-2 antibody on cell proliferation and the expression of various differentiation markers collectively suggest that cell-differentiating and antiproliferative actions are mediated through different molecular pathways.

The effects of the D5, Her-2 antibody and its IMC were evaluated on the growth of BT-474 cells transplanted in athymic mice. When given separately by intraperitoneal injection at an equivalent concentration to that used in the IMC, vehicle treatment, Her-2 antibody treatment, and D5 each failed to inhibit *in vivo* growth of BT-474 cells. Interestingly, dietary supplement of D5 showed significant inhibition on tumor growth. These results are in agreement with our previous findings in UISO-BCA-4 breast cancer cells.¹⁵ Furthermore, administration of IMC (once weekly) also inhibited the growth of BT-474 cells. We believe that the Her-2 antibody in IMC may simply act as a carrier protein to deliver D5 specifically to Her-2 expressing breast cancer cells. Thus, IMC is able to increase the intratumoral concentration of D5, which in turn inhibits the growth of breast cancer cells. Alternatively, D5 immunoconjugated to Her-2 antibody is stable and is retained by the tumor cells for a prolonged period, making it effective in inhibiting the *in vivo* growth of breast cancer cells. The results on how different administration routes of D5 (intraperitoneal vs. oral in the diet) affect BT-474 cell growth suggest that when D5 is administered unconjugated, it is retained by the cells for a shorter time and thus fails to show growth inhibition. Even though the relative weekly cumulative doses of D5 were similar (whether animals received it once a week by intraperitoneal injection or received it as a dietary supplement), the growth inhibitory action on BT-474 cells was seen only in the dietary group. These results suggest that D5 needs to be given frequently to achieve and maintain the intratumoral concentration necessary for tumor growth inhibition. Generally, a steady blood level of the compound is maintained when given continuously in the diet, and that in turn may help to maintain a sustained concentration of D5 available to tumor cells. It is possible that administration of D5 by the intraperitoneal route could rapidly generate a very high concentration (in the blood and tumor cells) that is nonetheless transient due to its metabolic clearance and which therefore fails to generate the D5 concentration (in the blood and tumor cells) necessary to inhibit the growth of BT-474 cells. Similarly, Her-2 antibody treatment alone failed to show growth inhibition, and even though it showed potent cell differentiation in *in vitro* assay, we also postulate that the cell-differentiating effect of the Her-2 antibody is probably transient and thus fails to inhibit cell proliferation *in vivo*.

The effect of IMC on *in vivo* tumor growth was similar to that observed in animals receiving a daily supplement of D5. However, it appears that IMC therapy is relatively safer than the dietary supplementation. *In vivo*, dietary supplement of D5 slightly increased serum calcium levels; however, IMC treatment did not show significant increase in serum calcium levels. Recently, vitamins (especially active metabolites of vitamin D, 1,25(OH)₂D₃, and its synthetic analogs) are known to have potent growth inhibitory and cell differentiating actions in various cancer cell types, including breast cancer. However, the use of vitamin D-related compounds for the treatment of breast cancer is hindered due to their hypercalcemic activity. Results from our study suggest that it is feasible to use known vitamin D metabolites or analogs with potent growth inhibitory or cell differentiating actions as a therapeutic agent, even though they are toxic, using the IMC approach described in our present study. Also, even though the Her-2 antibody used in our study has no direct clinical application, the concept of the IMC used in our study has potential therapeutic value. We believe that tumor cells positive for Her-2 receptor but

refractive to the growth-inhibitory effect of Her-2 antibody therapy could be targeted by D5-like vitamin D analogs using an IMC approach. In conclusion, the results from our present study identify a possible new, safe tumor cell-targeted therapy for the treatment of highly aggressive Her-2-overexpressing breast cancer. Further

studies are in progress using herceptin as a D5 carrier protein to determine whether D5 immunolinked to herceptin is more effective at inhibiting breast tumor growth and metastasis than herceptin treatment alone.

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AMERICAN RADIUM SOCIETY

Collaborating in Oncology

January 23, 2004

Srinivasan Vijayakumar, MD
University of California, Davis Department of Radiation Oncology
UC Davis Cancer Center
4501 X Street, G126
Sacramento, CA 95817

Dear Dr. Vijayakumar:

We are pleased to inform you that your poster listed below has been accepted for poster presentation at the 86th Annual Meeting of the American Radium Society, to be held May 1-5, 2004, at the Silverado Resort and Country Club in Napa Valley, California.

All posters will be displayed throughout the entire length of the meeting on an assigned 4'x4' tack board, which will be available at the Silverado.

By participating in the American Radium Society's 86th Annual Meeting, attendees will learn about the advances in diagnosis and treatment of cancer. The Scientific Program Committee is composed of physicians from several disciplines, and the choice program content and Faculty selections were made with the distinct purpose of providing multi-disciplinary detail of head and neck, prostate, breast, colorectal, lung and CNS cancer diagnosis, treatment, and outcome. ARS seeks to emphasize in attendees this unique multi-disciplinary perspective based on a broader fund of data, and not simply specialty-centered.

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education (ACCME) through joint-sponsorship of the American College of Radiology and the American Radium Society. The American College of Radiology (ACR) is accredited by the ACCME to provide continuing medical education for physicians. As an accredited body, the ACR is required to keep on file signed statements of financial disclosure and investigational or "off-label" use of medical devices, products, or pharmaceuticals. Please complete the enclosed statements and return them to the ARS office by February 9, 2004.

Enclosed you will find a registration brochure. Each presenter **must** register for the meeting. Please return your completed Meeting Registration to the ARS office either to the address above, or via fax to 708-687-1072. **Reserve your room early** in order to secure your reservation for your stay at the Silverado Resort.. A one-night deposit is required. The Tour Registration form should be returned to the address on the form.

If you have any questions regarding your presentation, please do not hesitate to call the ARS office at 708-687-1034.

Sincerely,

Bruce Haffty, MD
Scientific Program Chairman
Enclosures

Title: Clinical Trial Design in Chemoprevention Studies: Using a Vitamin D5 Analog Study as an Example

Please select Print from the file menu to print your Abstract.

American Radium Society 86th Annual Meeting

Filename: 250134

Presenting Author: Srinivasan Vijayakumar, MD

Contact Author: Srinivasan Vijayakumar, MD

Department/Institution: Department of Radiation Oncology, University of California, Davis

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Phone: 916-734-7888 **Fax:** 916-734-7076 **E-mail:** vijay@ucdavis.edu

Categories: Cancer Biology; Radiation Biology (including normal tissue effects); Urological;

Presentation Format: Poster

ARS Member: Yes

Send a membership application: No

Type of Study: Randomized

Values of comparative treatments:

Total cases in population: 40

Number evaluable: 40

Median follow-up: 2 years

Range of follow-up: 2 years

Will Discuss or Describe use of "off-Label" medical devices, Products, or Pharmaceuticals: Yes

More than 6 authors ("et al" will be published in program following the names listed below): No

Title: Clinical Trial Design in Chemoprevention Studies: Using a Vitamin D5 Analog Study as an Example

Srinivasan Vijayakumar, MD*, Rajendra Mehta, PhD, Rajeshwari Mehta, PhD, William Hall, MD, P Boerner and Laurel Beckett, PhD.

Body:

Purpose: There are significant design issues in chemoprevention studies, especially post radiotherapy (RT). Important aspects to be considered are serum and tissue intermediate biomarkers. After reviewing past studies, we designed a clinical trial and use it as an example to discuss the issues.

Vitamin D analogs have shown anti-tumor activity in breast and prostate cancer cell lines

and in murine models. However, the clinical use of such compounds is limited by hypercalcemic toxicity. The newly synthesized analog $1\alpha(\text{OH})\text{D}_5$ (1α -Hydroxy-24-ethyl-cholecalciferol) has shown anti-tumor activity at non-hypercalcemic concentrations in animals. This is a phase I/II safety/chemoprevention study to determine whether D5 can safely delay prostate cancer recurrence when administered after RT.

Methods: 40 randomized patients will receive either D5 or placebo, 12-60 months after completion of RT. Patients will receive baseline clinical staging, pre-treatment biopsy and serum PSA levels. Serum chemistries, albumin and PTH, and urine electrolytes will be obtained.

Subjects will be monitored using serum chemistries and albumin weekly in the first month. Individuals with stable calcium levels will then have weekly phone calls and monthly clinical assessments. Serum chemistries, albumin and PTH, and urine electrolytes will be obtained monthly. PTH will be monitored biannually. Individuals with stable calcium levels at 4 months will transition to a 4-month monitoring cycle, with chemistries, albumin and PTH, and urine electrolytes drawn immediately prior to a visit.

At the end of the study, subjects will receive final laboratory and clinical evaluations and undergo a prostate biopsy. Patients will receive 2 years of post-treatment follow-up.

Conclusions: We have designed a randomized Phase I/II study with end-of-study tissue biopsy, enabling assessment of tolerance, compliance, Quality of Life and intermediate bio-/molecular markers. The benefits of such a design will be discussed.

Disclosure: I have nothing to disclose.

Signature of Presenting Author:

Srinivasan Vijayakumar

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Appendix 5
OFFICE OF HUMAN RESEARCH PROTECTION
Administrative Review Checklist – Full Committee and Expedited Review Studies

Principal Investigator: Srinivasan Vijayakumar, M.D. Date of Review: _____

Items checked ✓ indicate a deficiency and must be addressed/corrected/revised

- ☐ Ensure PI has completed current IRB forms dated "6/2003".
- ☐ Ensure completion of the IRB approved educational program and test for all research personnel involved in the conduct of the study. Certifications must be current – on-line tutorial taken in 2002 or later.
- ☐ Attach copies of the sponsor's protocol, if sponsored by a pharmaceutical company
- ☐ Attach copies of the federal proposal/grant, if sponsored by federal funding
- ☐ Complete the Investigational Drug Information Record, if investigational drug(s) involved. IND number must be noted on the form.
- ☐ Complete the Investigational Device Form, if investigational device involved.
- ☐ If a Renewal, ensure the PI has submitted the progress report. Ensure all 20 points have been addressed and numbered individually with response.
- ☐ Ensure PI has completed **all pages** of the application form
- ☐ Ensure questions on HIPAA have been addressed on the first page of the application form.
- ☐ Attach copies of all advertisements, if applicable
- ☐ Attach copies of questionnaires and/or surveys, if applicable
- ☐ If a Renewal, attach copies of modifications approved by the IRB during the past year, if applicable
- ☐ If a Renewal, attach summary of adverse and serious adverse events in a table/chart format, if applicable
- ☐ Description of the Study:
 - ☐ Number of subjects – number must be consistent on application form, description of study and consent form.
 - ☐ All description of study headings must be addressed: ***purpose, methods, procedures, subject selection, risks, benefits, risk-benefit ratio, costs to subjects, disclosure of personal/financial interest, and resources.***

☐ Review Consent Form(s) as follows:

- ☐ Ensure the Bill of Rights and the Letter to the Research Participants (see OHRP web site for most current version) is attached as page 1 and 2 of the consent form.
- ☐ Review for standard format (UCD header, title of study, names and telephone numbers of Investigators, standard headings, and consent form version number at bottom of each page)
- ☐ Ensure all pages include page numbers
- ☐ Review for grammatical errors
- ☐ Ensure an initial line is on each page except the last page where the signature block appears
- ☐ Ensure the last page includes a signature block for the research subject and the investigator
- ☐ Ensure the number of subjects is addressed in the *Procedures* section
- ☐ Ensure the time commitment is addressed in the *Procedures* section
- ☐ Ensure *Confidentiality* section includes standard subpoena language
- ☐ Ensure Costs and Compensation are addressed and standard language is included (see model consent forms). If there is no cost or compensation it must state so.
- ☐ Ensure injury language is included in section entitled *Emergency Care and Treatment for Injury*
- ☐ Ensure the PIs name and telephone number and the OHRPs phone and address are listed in the *Questions* section

REMINDER: If you wish the OHRP to conduct the administrative review, only one copy marked "DRAFT" is required at this time. Do not send the original plus copies until you have corrected the deficiencies noted by the OHRP.

Date OHRP relayed comments/deficiencies to PI:

Principal Investigator:

Date:

I, _____ (signature required) attest that the protocol has undergone the above administrative review and that I have corrected the deficiencies, if any, identified by me or the OHRP. (8/2003)

**PROTOCOL FORM FOR RESEARCH
INVOLVING HUMAN SUBJECTS**

Protocol #: _____

Special
Requirements: _____

Reviewers: _____

- Full Committee Review

1st Review: _____ Action _____

2nd Review: _____ Action _____

3rd Review: _____ Action _____

OHRP Web Site: ovcr.ucdavis.edu/humansubjects
OHRP, Ambulatory Care Center, Suite 3870, UCDMC

Department ID #: _____

Principal Investigator: Srinivasan Vijayakumar, M.D. Employee ID #: 043-24-5133 University Position: Professor & Dept. Chair
Department: Radiation Oncology Telephone #: 916-734-7888 Fax #: 916-734-7076

Principal Investigator's E-Mail Address: vijay@ucdavis.edu Protocol Contact Person (name, telephone number and e-mail): Phil Boerner, 734-3981; Philip.boerner@ucdmc.ucdavis.edu

Title of the Research:
A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

X External, Sponsor Name: Department of Defense

Duration of the Study: 24 months

☐ Department Funding

Location of the Study: UCD Cancer Center

☐ Other, UC Source:

04-135

HIPAA:

X Yes ☐ No Will health information be obtained from the covered entity (a health care provider who bills health insurers, e.g., UCDHS)?
X Yes ☐ No Will the study involve the provision of healthcare in a covered entity, such as UCD Health System or Cowell Student Health Center?
X Yes ☐ No If the study involves the provision of healthcare, will a health insurer or billing agency be contacted for billing or eligibility?

If you answered "NO" to all three questions, you are not subject to HIPAA and do not need to address HIPAA in the Description of Study nor Page 4 of this form. If you answered "YES" to any of the questions above, you are subject to HIPAA and must address recruitment in the Subject Selection category (see Description of Study instructions) AND address Page 4 of this form.

SIGNATURES

Principal Investigator _____ Date 2/9/04
I certify that I have the appropriate credentials and privileges to conduct this study and that the facilities are adequate.

Faculty Advisor N/A Date 2/14/04
Required for student investigators

Department Chair _____ Date _____
I certify that the investigator has the appropriate credentials and privileges to conduct this study and that the facilities are adequate.

Dean of School _____ Date 2-17-04
Not required for A&ES or L&S studies

RECEIVED
SCHOOL OF MEDICINE
OFFICE OF SPONSORED
PROGRAMS
04 FEB 12 PM 2:28

OHRP Use Only: IRB Review and Approval

IRB Chair/Vice Chair _____ Approval Date _____

VC Research _____ Date _____

Protocol Expiration Date _____ Protocol Risk Level: Minimal Risk ☐ At Risk ☐

HIPAA: ☐ Waiver of Authorization (Form W) ☐ Recruitment Authorization (Form R)

Research Personnel

Please list **ALL** research personnel involved in the conduct of this study. All personnel must complete the IRB approved educational program on the protection of human subjects, and provide to the OHRP Office the certification and attestation forms verifying completion of the courses. **The IRB will not review a study without such forms on file for all research personnel.** The employee ID number is required. This number is **not** the employee's social security number. Please see your Department Manager for assistance.

Name	Employee ID #	Title	Department
Srinivasan Vijayakumar, MD	043245133	Professor & Chair	Radiation Oncology
Samir Narayan, MD	372945186	Assistant Professor	Radiation Oncology
Ralph W. de Vere White, MD	031107253	Professor & Chair	Urology
Janice Ryu, MD	102708062	Associate Professor	Radiation Oncology
Paul Gumerlock, MD	826697583	Adjunct Professor	Hematology & Oncology
Laurel Beckett, PhD	839098225	Professor	Epidemiology & Preventive Medicine
Ralph Green, MD	659794200	Professor & Chair	Pathology
William Baker, Jr, MD	N/A	Chief, VA Mather	Urology, VA
Rajendra Mehta, PhD	N/A	Professor	Surgical Oncology, UIC
Rajeshwari Mehta, PhD	N/A	Associate Professor	Surgical Oncology, UIC
Alan Diamond, PhD	N/A	Professor & Head	Human Nutrition, UIC
Cathy Hollister	169091386	Research Coordinator	Radiation Oncology
Cheri Koppe	735079816	Research Coordinator	Radiation Oncology

Who Obtains Informed Consent

I, Srinivasan Vijayakumar, M.D. do hereby attest that the following research personnel have read the protocol,
Principal Investigator's Name

understand the study, and are fully knowledgeable of ALL details of the protocol and are able to answer ALL questions from research subjects such as risks and alternative treatments and therapies. Such personnel may obtain informed consent from research subjects along with the principal investigator.

Name	Title	Department
Srinivasan Vijayakumar, MD	043245133	Professor & Chair
Samir Narayan, MD	372945186	Assistant Professor
Janice Ryu, MD	102708062	Associate Professor

Principal Investigator's Attestation:

Signature of Principal Investigator

Date

2/8/04

INSTRUCTIONS FOR COMPLETING A HUMAN SUBJECTS PROTOCOL

I. DESCRIPTION OF STUDY: The following categories make up the Description of Study:

- PURPOSE, METHODS, AND PROCEDURES:** describe in detail the purpose, research methods and procedures of the study. Address how you will monitor this study to ensure that the study is being conducted according to the protocol. Clarify whether you will have a Data Safety Monitoring Board to conduct such monitoring.
- SUBJECT SELECTION:** indicate how subjects will be recruited, from where and when, and how many. Address the inclusion and exclusion criteria. When vulnerable populations are involved, describe why they are necessary. If women or minorities are excluded, explain reasons for exclusion. If you are using protected health information to recruit subjects address the following points: a) address how you plan to protect the identifiers used to identify potential subjects. b) address how and when you plan to destroy these identifiers. c) Insert the following standard language: "No identifiers, used for recruitment purposes, will be disclosed to a third party except as required by law or for authorized oversight of the research project." The IRB will issue you a *Waiver of Authorization for Recruitment* upon successful review and approval of your responses.
- RISKS:** describe any potential risks to subjects, physical, psychological, social or legal. Assess the likelihood and seriousness of those risks. If the methods of research create potential risks, describe other methods, if any, that were considered and why they will not be used. Address procedures for maintaining confidentiality if confident it represents a risk. Explain your reporting mechanism for reporting adverse and serious adverse events to the IRB.
- BENEFITS:** describe the anticipated benefits of the research to the individual subjects, to the particular group or class from which the subject population is drawn. If there is no direct benefit to the subject, state so.
- RISK-BENEFIT RATIO:** assess the relative weights of the study's risks and benefits.
- COSTS TO SUBJECTS:** if the investigation involves the possibility of added expense to the subject or to a third party, such as an insurer (e.g., longer hospitalization, extra laboratory tests, travel) indicate how this is justified. If there is compensation for unpleasant or risky procedures, compensation must be staged.
- DISCLOSURE OF PERSONAL AND FINANCIAL INTEREST IN THE RESEARCH STUDY AND SPONSOR:** the investigator must disclose any personal and financial interests in the research as well as the extent of personal and financial interest in the entity. If the investigator anticipates use of therapeutically removed tissue, include this information in the Description of Study.
- RESOURCES:** Address how you will ensure that you have the appropriate resources (study personnel and facilities) to conduct this study according to the protocol. Describe the role of each member of your study personnel and their FTE appointment to this research study.

II. INFORMED CONSENT OF SUBJECTS:

EXPERIMENTAL SUBJECT'S BILL OF RIGHTS (Bill of Rights): the Bill of Rights must be given to subjects prior to consent. Investigators are required to secure the subject's signature and the date signed. The signed copy, along with their signed copy of the consent form, must be provided to each subject. Investigators are required to incorporate the Bill of Rights as page one of the consent form. Review the Model for medical studies and the Model for social and behavioral studies and select as appropriate for your study.

LETTER TO RESEARCH SUBJECTS - WEB SITE FOR PROSPECTIVE AND CURRENTLY ENROLLED SUBJECTS: the letter, developed by the OHRP, must be provided to all subjects along with the Bill of Rights and the Consent Form. Investigators are required to incorporate the Letter as page two of the consent form.

INFORMED CONSENT: review the Model Consent Forms and select and develop as appropriate for your study. See the following Models: Standard Model Consent Form, Venipuncture Model Consent Form, Social and Behavioral Model Consent Form, Treatment Use Model Consent Form, and Specimen Model Consent Form.

III. SPECIAL REQUIREMENTS: check where appropriate and submit 25 copies with your protocol submission. **Please note, some documents may require fewer copies.** Please review the requirements carefully.

- ☒ Investigational Drug Study - attach 4 complete copies of drug company protocol. Also complete the Investigational Drug Information Record, Form 710 B, and attach 25 copies of this form.
- ☐ Investigational Device - complete and attach Form 710A2.
- ☐ Approved Drug New Use - complete and attach Form 710B.
- ☒ Federally Sponsored Studies - submit 2 complete copies of the federal proposal/grant.
- ☐ Interviews (phone or in person) - attach script.
- ☐ Surveys/questionnaires - attach surveys and questionnaires.
- ☐ Studies involving minors in school settings - attach approval letter from the School Principal or approval from the School District Office.
- ☐ Advertisements, press releases, in-class announcements, or bulletin board announcements - attach proposed notice.
- ☐ Incomplete disclosure - include justification.
- ☐ Radiation/Radioactive materials use (including diagnostic x-rays/fluoroscopy). Attach Radiation Use Committee approval letter (telephone 734-3355).
- ☒ Cancer Clinical Trials - all cancer-related clinical trials must be reviewed by the Cancer Center Scientific Review Committee **PRIOR** to submission to the IRB. Contact the Scientific Review Committee at 734-8053 for information and requirements.

IV. TOTAL NUMBER OF COPIES REQUIRED FOR SUBMISSION TO THE OHRP: original plus 24 copies for the IRB Clinical Committees. Original plus 14 copies for the IRB Social & Behavioral Committee. Submit to the OHRP, Ambulatory Care Center, Suite 3870, UCDMC.

V. ESTIMATED NUMBER OF SUBJECTS: check all that apply (a subject may fit into more than one of the categories below)

<u>20</u> Patients as experimental subjects	<u>20</u> Patients as controls	_____ Persons whose first language is not English
_____ Students	_____ Prisoners	_____ Pregnant women or fetuses
_____ Mentally disabled	_____ Psychiatric patients	_____ Normal adult volunteers
_____ Minors (18 and under)	<u>40</u> TOTAL NUMBER OF SUBJECTS	

HEALTH INSURANCE PORTABILITY AND ACCOUNTABILITY ACT

HIPAA WORKSHEET

INVESTIGATORS ARE ADVISED THAT THE PRIVACY OFFICIAL AND THE INSTITUTIONAL REVIEW BOARD MAY AUDIT THIS STUDY FOR VERIFICATION AND COMPLIANCE WITH THE INFORMATION BELOW.

PROTECTED HEALTH INFORMATION - Check ☒ all that apply unless the data will be de-identified or the data will be collected under a data use agreement:

<input checked="" type="checkbox"/> Any Part of the Medical Record	<input checked="" type="checkbox"/> Laboratory Reports	<input checked="" type="checkbox"/> Emergency Medicine Center Reports
<input checked="" type="checkbox"/> Billing Statements	<input checked="" type="checkbox"/> Dental Records	<input checked="" type="checkbox"/> History & Physical Exams
<input checked="" type="checkbox"/> Pathology Reports	<input checked="" type="checkbox"/> Operative Reports	<input checked="" type="checkbox"/> Diagnostic Imaging Reports
<input checked="" type="checkbox"/> EKG	<input checked="" type="checkbox"/> Radiology Reports	<input checked="" type="checkbox"/> Consultations
<input checked="" type="checkbox"/> Progress Notes	<input checked="" type="checkbox"/> Radiologic or MRI Images	<input checked="" type="checkbox"/> Outpatient Clinic Records
<input checked="" type="checkbox"/> Genetic Testing	<input checked="" type="checkbox"/> Discharge Summary	<input checked="" type="checkbox"/> Psychological Tests
<input checked="" type="checkbox"/> Drug and Alcohol Abuse, Diagnosis or Treatment	<input checked="" type="checkbox"/> HIV/AIDS Testing Information	<input checked="" type="checkbox"/> Mental Health Diagnosis or Treatment

PLEASE CHECK ONE OF THE FOLLOWING:

☒ Subjects sign consent form upon enrollment in the study (Your study requires the use of the Research Authorization Form. See web site address: <http://compliance.ucdmc.ucdavis.edu/guidance/privacy/resauth.html> for the Form, instructions and requirements on the use of the Research Authorization Form. Please note: the IRB does not require submission of the Research Authorization Form with your IRB protocol application forms. However, you are required to provide the Research Authorization Form to the research subjects, in addition to the consent form, as instructed on the UCD Compliance Web Site. Your study may be audited for compliance with this requirement).

☐ All data received for the study is de-identified (see web site address: <http://compliance.ucdavis.edu/guidance/privacy/deident.html>).

☐ Data is collected under a data use agreement and is a limited data set (see web site address: <http://compliance.ucdmc.ucdavis.edu/guidance/privacy/limdata.html>).

☐ Subjects do not sign a consent form (see instructions below for studies that do not include a written consent form).

WEB SITE ADDRESS FOR GENERAL INFORMATION: <http://compliance.ucdmc.ucdavis.edu/guidance/privacy/>

STUDIES WHICH DO NOT INCLUDE A WRITTEN CONSENT FORM:

If your study does NOT include a written consent form (e.g., you are submitting a Statement of Exemption or you are submitting a protocol for either Full Committee or Expedited Review and you are requesting that the consent form be waived by the IRB), OR a Data Use Agreement has been issued to you by the covered entity (see the UCDHS Compliance Officer) address questions 1-5 below. Your responses must be numbered according to the questions below and be attached to this Worksheet. Submit your response to the IRB along with your protocol application forms. The IRB will assess whether a *Waiver of Authorization* (notification to the research subjects that you are using their PHI is not required) is appropriate. If so, the IRB will issue you the document, *Waiver of Authorization*, along with the other IRB approval forms at the time of IRB approval of the study.

1. Please provide details of why the research could not practicably be conducted without access to and use of the PHI.
2. Do you have an adequate plan to protect identifiers from improper use and disclosure? Please provide details.
3. Do you have an adequate plan to destroy all identifiers at the earliest opportunity consistent with the conduct of the research? Please explain if there is a research or health justification for retaining identifiers or if retention of identifiers is otherwise required by law.
4. Insert the following standard language: PHI will not be reused or disclosed to any other person or entity, except as required by law or for authorized oversight of the research project.
5. Provide an expiration date for the disclosures or if this is a therapeutic study, state far enough in the future to satisfy the sponsor's need for data/information.

University of California, Davis
Office of Human Research Protection
INSTITUTIONAL REVIEW BOARD

INVESTIGATIONAL DRUG INFORMATION RECORD
CLINICAL AND PHYSIOLOGICAL RESEARCH

INSTRUCTIONS: Please provide 20 copies of the following information for each investigational drug or for each new use for an approved drug. Be sure to attach any drug company protocol, report of animal studies, toxicity, and prior use in humans. It is hospital policy that the pharmacy is responsible for storing and distributing all investigational drugs. Please make arrangements prior to initiating investigations by contacting the UCDMC Pharmacy Investigational Drug Service.

Generic Drug Name/Strength/Dosage Form: 1 α -Hydroxyvitamin D₅; the chemical name for it is 1 α -Hydroxy-24-ethyl-cholecalciferol [1 α (OH)D₅] / **Strength:** 10 μ g / **Dosage form:** oral capsule

Synonyms: D5 **IND #** 56,509-000

Manufacturer: Merrifield Pharma, Inc. **NDA #** pending

Filing Date: December 1998

Person and/or Institution who filed this IND: University of Illinois, Chicago / Dr. Tapas K. Das Gupta, P.I.

Drug Source (if other than manufacturer): N/A

Sponsor of Study:

Department of Defense: Congressionally Directed Medical Research Programs

Project Title: A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D₅ Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

Principal Investigator:(Name, University position, addresses, phones)

Srinivasan Vijayakumar, M.D.,

Professor and Chair, Department of Radiation Oncology, 4501 X Street, Suite G126, UCDMC

Is this the first time this drug has been used in humans? ☒ yes ☐ no

☒ PHASE I ☒ PHASE II ☐ PHASE III ☐ APPROVED DRUG, NEW USE

Identification of Study Design:

☐ Single Blind ☒ Double Blind ☐ Open Trial ☐ Cross Over
☒ Placebo-Control ☐ Drug Control ☐ Other

If Other, please describe:

Indications for use of drug:

chemoprevention of prostate cancer

Usual Dosage Range:

the standard dose of D5 will be 10 µg per capsule, taken once a day, for two years. Dose de-escalations are possible if toxicity occurs (see next section).

Protocol Treatment Regimen:

The study medication will be dispensed monthly by the research nurse. All patients will receive a one-month supply of either D5 or the placebo at their monthly visit with the research nurse, along with the pill diary form to record their medication use. Both of the study arms will follow the same schedule of drug administration. As stated in the protocol, the standard dose of D5 will be 10 µg per capsule, taken once a day. It will be recommended to patients that they maintain a low calcium diet and avoid calcium-containing medications, such as Tums.

At each follow-up visit, an assessment of patient medication compliance will be made and recorded in the patient's medical record. Compliance will be recorded as the percentage of pills taken. To help in the assessment of compliance, it is required that patients keep a pill diary record (using the form provided to them) of their daily pill consumption. Prior to starting treatment, the patient will be provided with and instructed in the proper use of a pill diary. The patient will be instructed to return this diary at specified intervals during treatment and at each follow-up visit. This record will be checked for compliance by the investigator. The diary will be retained in the patient's record. The diary will act as source documentation. Patients who are non-compliant with diary use will be re-instructed in the use of the diary.

Toxicity-Based Dose Modification Schedule for D5**Toxicity: Grade 3 or 4**

1st appearance: The patient will go on a drug holiday for one month or until the toxicity has been resolved to grade 0-1, whichever is longer, then continue at 50% of starting dose (i.e., 5 µg per day)

2nd appearance: Interrupt for one month or until resolved to grade 0-1, whichever is longer, then continue at 50% of previous dose (i.e., 2.5 µg per day)

3rd appearance: Interrupt for one month or until resolved to grade 0-1, whichever is longer, then continue at 50% of previous dose (i.e., 1.25 µg per day)

4th appearance: Discontinue treatment permanently

Route(s) of Administration:

oral (capsule)

Mechanism of Action: The exact mechanism by which Vitamin D5 exerts its antitumor activity is not known and remains under investigation. Several mechanisms that have been proposed to explain this activity include: Modulation of signal transduction and oncogene expression, Inhibition of DNA synthesis, Induction of cell cycle arrest, Up-regulation of cyclin-dependent kinase inhibitors, Decreased E2F transcriptional activity, and Inhibition of angiogenesis and tumor invasion via reduction in type IV collagenase production.

Possible Side Effects: Hypercalcemia: symptoms include loss of appetite, nausea, vomiting, abdominal pain, constipation, and other symptoms. There may also be unknown effects since the study drug is a newly synthesized analog of vitamin D.

Precautions, Warnings, and Contraindications: The primary toxicity associated with Vitamin D analogs such as Vitamin D5 is hypercalcemia. Hypercalcemia is an elevation in the concentration of calcium in the blood. The symptoms of hypercalcemia include fatigue, loss of appetite (anorexia), depression, anxiety, weight loss, nausea and vomiting, hot flashes, itching abdominal pain, constipation or diarrhea, excessive urination, dehydration, and fainting. Dehydration can be severe enough to cause reduced blood flow to the kidneys and possible kidney damage. Additionally, hypercalcemia may result in increased excretion of calcium into the urine, a condition called hypercalciuria, which can result in the formation of kidney stones. Additional

theoretical toxicities include increased bone reabsorption leading to osteoporosis. Additional complications occurring with severe hypercalcemia include stomach or intestinal ulcers, pancreatitis (a severe inflammation of the pancreas), organic brain dysfunction (confusion, cognitive impairment, memory loss), calcification of heart valves, coronary artery calcification, myocardial calcification (calcification of heart muscle possibly leading to heart failure), hypertension, muscle calcification, muscle weakness, and formation of calcium plaques in the eye.

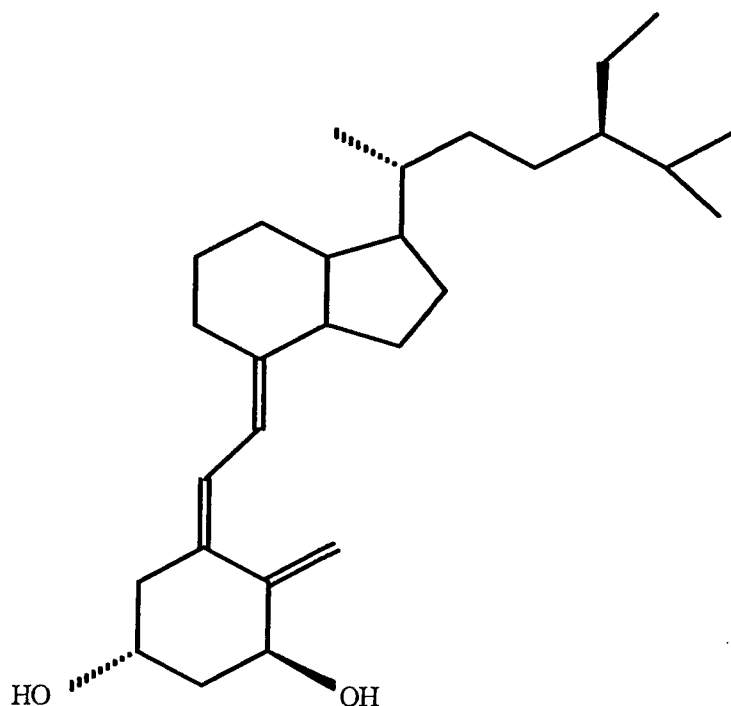
Individuals with known hypercalcemia, known hyperphosphatemia, history of hypercalcemia, renal impairment (GFR <60mL/min/1.72m²), history of calcium type kidney stones, known osteoporosis, hyperparathyroidism, sarcoidosis, or distal renal tubular acidosis (RTA type1) should avoid taking vitamin D5 or other vitamin D analogs. Individuals using calcium or vitamin D supplements either singly or in multivitamin form, or calcium based anti-acid medications should avoid combining these medications with vitamin D5 or other vitamin D analogs. Because D5 does not yet have FDA approval (the FDA needs a few more clarifications regarding the stability of the compound using more than one method; this is in progress), formal FDA warnings have not been issued.

IV Drug Formulation/Preparation/Stability: This medicine will only be used in oral form. The analog 1 α (OH)D₅ was synthesized by ConQuest, Inc. (Chicago, IL) under Good Manufacturing Practice for the Phase I/II clinical trial for breast cancer patients and is available from Merrifield Pharma, Inc. (Westmont, IL) for the present study. We also have completed (as a subcontract to IIT Research Institute, Chicago) preclinical toxicity studies in two species. We will purchase 1 α (OH)D₅ from Merrifield Pharma, Inc. (ConQuest, Inc. was sold to United Therapeutics in 2000 and no longer manufactures D5.) Meeting the prerequisites for using a compound in a clinical setting is very crucial for the success of the project. The current study therefore can be implemented clinically without any delay, once FDA approval is obtained.

Physical, Chemical and Pharmaceutical Properties and Formulation

Chemical Information

1 α -Hydroxyvitamin D5 Structural Formula



Chemical Name: 1 α -Hydroxyvitamin D5 is a structural analog of vitamin D5. The chemical name for it is 1 α -Hydroxy-24-ethyl-cholecalciferol [1 α (OH)D₅]

Synthesis: The compound has been synthesized by Dr. Raju Penmasta, Merrifield Pharma, Inc., Westmont, IL according to the Good Manufacturing Practice guidelines.

Molecular formula: C₂₉H₄₈O₂

Molecular Weight: 428.6

Physical form: White powder

Solubility: It is insoluble in water but highly soluble in ethanol.

Purity: The acceptable limit for the purity of the substance is 95-100%, and the analytical method used to assure the identity and purity of the compound is reversed-phase HPLC. The compound, 1 α -hydroxyvitamin D5, was separated on a C18-reversed phase 75x 4.6 mm, 3.5 micron column using a mobile phase of 90% acetonitrile in water. 1 α -Hydroxyvitamin D5 was separated with a flow rate of 1 ml/min and monitored at 265 m μ . It was eluted with the retention time of 35 min.

The compound is being formulated in the form of an oral capsule. The concentration in each capsule will be created according to the protocol approved for the Phase I clinical trial. This will be comparable to the oral capsule given to animals in preclinical toxicity studies under Good Laboratory Practice guidelines. The capsules for each dose level will be prepared according to the dosage schedule at the time of the initiation of the study (Table 1). This will be prepared within the Pharmaceutical Science Department in the School of Pharmacy at the University of Illinois. All the inactive ingredients in the capsule will be standard pharmaceutical components, which comply with pharmacopeal guidelines. The capsules will be stored in a freezer to avoid degradation of vitamin D.

Administration or Dispensing Instructions (Including warnings to be given to patients):

See "Protocol Treatment Regimen" section above, and also "Precautions, Warnings, and Contraindications" section above.

Compatibility/Incompatibility:

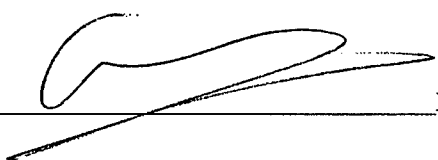
D5 is compatible with most medications. It is not recommended with medications that may result in hypercalcemia. Individuals using calcium or vitamin D supplements either singly or in multivitamin form, or calcium based anti-acid medications should avoid combining these medications with vitamin D5 or other vitamin D analogs.

NOTE: If available, attach any drug company protocol or information that will assist the human subjects review committee in their review.

List of attachments: None

Signature of Investigator: _____

Date: _____



2/9/04

Cancer Center Scientific Review Committee
APPROVAL FORM

12/11/04

SRC Meeting Date: _____

Principal Investigator: Srinivisan Vijayakumar, M.D.

Disease Site: Genitourinary

Disease Sub site: Prostate

Study Number: UCDCC#141

Sponsor: Department of Defense

Protocol Title: A Phase I/II Double-Blinded, Randomized Clinical Trial To Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1a-Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

Total Target Accrual: 40

UCD Target Accrual: 40


Does protocol involve Lawrence Livermore National Laboratory (LLNL)?

- ☐ Yes – copy of form, SRC minutes and comments sent to LLNL IRB
☒ No

Voting Outcome:

- ☒ Approved
☐ Approved with recommendations (explain below)
☐ Approved with mandatory revisions - resubmission to SRC required (explain below)
☐ Disapproved (explain below)
☐ Tabled (to be reconsidered at next SRC meeting)
☐ Withdrawn

Explanation/Additional Comments:



Co-Chair Signature (Primo Lara, M.D.)

2/9/04

Date

NOTE: Original must be included in IRB submission package

Title of Study: A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

1. PURPOSE, METHODS, AND PROCEDURES

This is a phase I/II safety/chemoprevention study to determine whether taking a non-toxic Vitamin D analog, D5, can safely prevent prostate cancer recurrence when administered after radiation therapy (RT). Vitamin D analogs have shown anti-tumor activity in breast and prostate cancer cell lines and in murine models. However, the clinical use of such compounds has been limited by hypercalcemic toxicity. The newly synthesized analog 1 α (OH)D5 (1 α -Hydroxy-24-ethyl-cholecalciferol) has shown anti-tumor activity at non-hypercalcemic concentrations in animals. Based on their preliminary research, investigators in this study believe D5 can be given in effective doses without causing harmful side effects.

Forty randomized patients will receive either D5 or placebo, 12-60 months after completion of RT (20 patients/arm). The study population will consist of intermediate and high-risk patients. Patients will have received baseline clinical staging and pre-treatment biopsy prior to their radiation treatment. Patients will receive clinical assessment and PSA on entering into the study. Serum chemistries, albumin and PTH, and urine electrolytes will also be obtained.

During the study patients will be closely monitored for hypercalcemia as well as other potential toxicities. Doses of D5 will be lowered or stopped if toxicities occur. Subjects will be monitored using serum chemistries and albumin weekly in the first month. Individuals with stable calcium levels will then have weekly phone calls and monthly clinical assessments. Serum chemistries, albumin, PTH, and urine electrolytes will be obtained monthly. PTH will be monitored biannually. Individuals with stable calcium levels at 4 months will transition to a 4-month monitoring cycle, with chemistries, albumin, PTH, and urine electrolytes drawn immediately prior to a visit.

At the end of the study, subjects will receive final laboratory and clinical evaluations and undergo a prostate biopsy. Study endpoints include differences between study groups in drug tolerance and compliance, toxicity, quality of life, biomarker presence and proportion of patients developing PSA-based biochemical failure or clinical failure. Biopsies will be evaluated for selective markers indicating any benefit of D5 in decreasing the recurrence of prostate cancer. Patients will continue to be followed for any clinical recurrences or toxicity as part of their usual cancer care.

Monitoring of the Study

The UCD Data Safety and Monitoring Committee will review the data at least every six months and evaluate the results. The study will be conducted according to Good Clinical Practice (GCP) guidelines. GCP is a standard for the design, conduct, performance, monitoring, auditing, recording, analysis, and reporting of clinical trials. The Good Clinical Practice Program is the

focal point within FDA for Good Clinical Practice issues arising in human research trials regulated by FDA. Per HSRRB requirements, a medical monitor, Dr. Rachel Chou, is assigned to this study. The medical monitor is required to review all serious and unexpected adverse events.

2. SUBJECT SELECTION

Recruitment and Informed Consent

40 subjects will be selected from eligible patients seen in the Radiation Oncology clinic. Potential subjects will be patients from the clinics of the study investigators. The investigators will make the initial contact and will assess the inclusion/exclusion criteria for potential subjects using interviews. The discussion that the investigators will have with potential subjects will closely follow the text of the consent form. The patient and his family will be given a consent form to take home and read, and will be encouraged to write down their questions. The patients will also receive a copy of this protocol. During the next patient visit approximately one week later, the consent form will be discussed further, and the potential subjects will be asked to confirm that they have read the description of the study. They also will be able to discuss the study with their doctors until all questions are answered. Potential subjects will be asked to state that they understand: (1) that the study is to determine whether or not the treatment is effective and tolerated, as well as how effective it is; (2) that their participation is voluntary; and (3) that they know enough about the purpose, methods, risks, and benefits of the study to judge that they want to participate. Each potential subject must be able to provide informed consent, which will be obtained by the investigators.

Inclusion Criteria

This study is for men who had received radiotherapy with curative intent. The patients must have had non-metastatic prostate cancer. They should have been staged by standard procedures, including:

- Digital Rectal Examination and documentation of the pre-RT findings in a AJCC Staging Sheet
- Pre-treatment biopsy and a report of the grade of the lesion
- Pre treatment PSA levels (must be between 2 and 8 at the time of registration)
- Bone Scan if the PSA level was over 15 ng/ml at the time of diagnosis

Radiotherapy:

- Should have been completed within 5 years from the date of registration, but not within the immediate twelve months.
- Could have been external beam RT [XRT] alone, XRT with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], brachytherapy alone, brachytherapy with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], or a combination of XRT and brachytherapy [again, if neoadjuvant hormonal therapy was given, it should have been for a duration not exceeding 12 months]

There should have been no evidence of metastatic disease at the time of diagnosis. There should be no evidence of metastatic disease at the time of registration. The PSA should have been stable [no more than 0.75 ng/ml variation in the PSA measurements], with at least 3 measurements within 12 months prior to the date of registration. The Karnofsky Performance Status [KPS] should be 80% or more. Patients have to sign an informed consent. They should be able to understand and consent in a fully informed document.

Patients should belong to Group II or III (intermediate or high-risk) based on T-stage, Gleason Sum and PSA criteria. The age range of the subjects will be from 18 to 65+ years of age. There are no medications and/or treatments, other than those listed in the inclusion/exclusion criteria, which study subjects must avoid due to the study medication.

Exclusion Criteria

1. Patients with metastatic disease.
2. Patients with a rising PSA as defined by the American Society for Therapeutic Radiology (ASTRO) criteria of three consecutive increases in PSA. PSA doubling time must be ≤ 6 months.
3. Patients who are on Androgen Deprivation Therapy.
4. Patients who are on 5-alpha reductase inhibitors such as Proscar. If they were on such therapy and discontinued at least 12 months prior to randomization, then they are eligible.
5. Patients with KPS less than 80%.
6. Patients with co-morbidities that lead to life expectancy of less than 5 years.
7. Patients who are unable to sign an informed consent.
8. Patients with simultaneous or second malignancies within 5 years of registration.
9. Patients who had prostatectomies as part of treatment for prostate cancer or other conditions [for example, Abdomino-Perineal resection for rectal cancer].
10. PSA at registration exceeding a value of 10 ng/ml or less than 2 ng/ml.
7. Patients who are considering fathering children.
8. Patients who are unable to swallow and retain oral medicine.
9. Patients who would require a consent form that has to be translated into another language (i.e., a language other than English).
10. Patients with existing hypercalcemia.
11. Patients with existing hypercalciuria.
12. Patients with existing hyperparathyroidism.
13. Patients with existing sarcoidosis.
14. Patients with existing type distal renal tubular acidosis (type 1 RTA).
15. Patients with existing osteoporosis.
16. Patients with existing renal insufficiency
17. Patients with a history of hypercalcemia while using vitamin D or vitamin D analogs.
18. Patients with a history of calcium containing kidney stones.
19. Patients with a history of hypercalemia related pancreatitis.

Women and Minorities as Study Subjects

Since this study concerns cancer of the prostate, women are not included in this study. Members of minorities are included.

Anonymity of Study Subjects

The anonymity of the study subjects will be maintained. In study records, subject names will not be used. Only initials will be used. No social security numbers will be used. Study coordinators will maintain a tracking book and be given a case study number for each study subject. *No identifiers, used for recruitment purposes, will be disclosed to a third party except as required by law or for authorized oversight of the research project.*

Any study records are going to be kept in a secure, locked cabinet in the Clinical Trials office. All of the University's data is password protected and only employees associated with the study will have access to them. Per University policy, study records will be maintained for 10 years.

3. RISKS

There are very few risks to subjects. Although preliminary studies have indicated a relative safety of the study medication, one of the purposes of this study is to see whether there are any unexpected side effects from it. One of the known side effects of Vitamin D, when taken in excess or when the potent analogs are used, is an increase in blood calcium levels, known as hypercalcemia. Symptoms of hypercalcemia include loss of appetite, nausea, vomiting, abdominal pain, constipation, as well as other symptoms. There may also be unknown effects since the study drug is a newly synthesized analog of vitamin D.

There are a number of potential risks and discomforts that subjects will be made aware of before they consent to participate. Subjects will be informed of any significant new findings developed during the course of the research that could affect their willingness to continue participation. The investigational agent to be used in this study is not approved by the Food and Drug Administration (FDA) for commercial use; however, FDA has permitted its use in this research study.

Another risk is the release of information from their health records, which may be necessary for investigators to obtain along with their specimens. Investigators will protect subjects' records so that their name, address, and phone number will be kept private.

Adverse Events Reporting

Adverse Events (AEs) for investigational agents will be submitted to CTEP via the web-based Adverse Events Expedited Reporting System (AdEERS).

In addition, adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (301-619-2165) (non-duty hours call 301-619-2165 and send information by facsimile to 301-619-

7803). A written report will follow the initial telephone call within 3 working days. Address the written report to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

It is the responsibility of UCD to report all adverse events to the Investigational Drug Branch (IDB), Division of Cancer Therapy (DCT) via AdEERS or the expedited adverse event report Single Agent or Multiple Agents paper templates (available on the CTEP Home Page, <http://ctep.cancer.gov>). Reports are to be submitted within the timeframes specified.

In addition, adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (see above).

AEs to be Reported to NCI Using AdEERS, and to USAMRMC include:

- all grade 4 and 5 AEs, including unrelated or unlikely unexpected and expected events
- any unexpected grade 2-5 AE (an expedited report is required for unexpected grade 2 and 3 AEs with an attribution of possible, probable or definite)
- any death within 30 days of drug administration
- any hospitalization (or prolongation of existing hospitalization) for medical events equivalent to CTC grade 3, 4, 5

Questions regarding AE reporting will be directed to the Clinical Research Associates, Cheri Koppe (916-734-3604, beeper 916-762-1601) or Cathy Hollister (916-734-8814, beeper 916-762-6282).

4. BENEFITS

As subjects will be randomized to treatment and control groups, ~50% of participants will receive D5. Those subjects would directly benefit from the hypothetical reduction in prostate cancer recurrence resulting from D5. The possible benefit is that vitamin D5 may be found to be an effective drug in preventing prostate cancer recurrence.

5. RISK-BENEFIT RATIO

This study poses minimal risk to participants and large potential benefit to future prostate cancer patients. Vitamin D5 may represent significant preventive treatment and ultimately provide direct benefit, measurable in reduced recurrence rates, in the ~50% of participants randomized to receive D5 treatment. As the theoretical risks to the administration of vitamin D5 are low and adequate steps have been undertaken to recognize and manage these risks, the treatment arm is at low risk in this study. The benefits of undertaking this study of vitamin D5 far outweigh the risks.

6. COSTS TO SUBJECTS

Subjects will not be charged or paid to participate in the study. The study medications will be provided free of cost. The routine blood tests that are part of their regular follow-up will be paid by either the patient's insurance company or by the patient. Subjects will not be charged for any of the blood tests that are specifically designed for the study.

7. DISCLOSURE OF PERSONAL AND FINANCIAL INTEREST IN THE RESEARCH STUDY AND SPONSOR

The principal investigator, co-investigators and sponsoring agency, the Department of Defense, have no personal or financial interests in this research study.

8. RESOURCES

Adequate funds have been allotted for personnel, consultants, subject-related costs, and other expenses. Specifically:

Srinivasan Vijayakumar, M.D. (5% FTE on the project), the PI for this project, is responsible for the overall project and the clinical protocol, which will include all aspects of the radiation therapy and treatment with vitamin D5, follow-up, and pathology as well as clinical chemistry.

Ralph deVere White, M.D. (2% FTE) Dr. deVere White will serve as Urologist on the project, assisting Dr. Vijayakumar with the clinical studies and obtaining biopsies

Research Nurse (TBN) (25% FTE). A nurse will assist Dr. Vijayakumar with the clinical studies.

Laurel Beckett, Ph.D., Statistician (1% to 1.5%). Dr. Beckett will assist with the experimental design, sample size, and statistical analyses. She will be used on an as-needed basis.

The following co-investigators will spend less than 1% of their time on the project:

Ralph Green, M.D., Pathologist. Dr. Green will collaborate on the project for the purposes of identification of PIN and other pathological conditions.

Samir Narayan, M.D.; Janice Ryu, M.D.; William Baker, M.D. These co-investigators will enroll patients into the clinical trial.

Paul Gumerlock, M.D. Dr. Gumerlock will assist with this study as it relates to the genetics of prostate cancer.

Rajendra Mehta, Ph.D. and Dr. Rajeshwari Mehta, Ph.D. These two co-investigators will conduct preliminary studies with D5, share their expertise in developing the appropriate doses of D5 for humans, and analyze data from the project.

UNIVERSITY OF CALIFORNIA, DAVIS
EXPERIMENTAL SUBJECT'S BILL OF RIGHTS
(Medical Research Studies)

The rights below are the rights of every person who is asked to be in a medical research study. As an experimental subject, you have the following rights:

- 1) To be told what the study is trying to determine.
- 2) To be told what will happen to you and whether any of the procedures, drugs, or devices is different from what would be used in standard practice.
- 3) To be told about the frequent and/or important risks, side effects, or discomforts of the things that will happen to you for research purposes.
- 4) To be told if you can expect any benefit from participating and, if so, what the benefit might be.
- 5) To be told the other choices you have and how they may be better or worse than being in the study.
- 6) To be allowed to ask any questions concerning the study, both before agreeing to be involved and during the course of the study.
- 7) To be told what sort of medical treatment is available if any complications arise.
- 8) To refuse to participate or to change your mind about participating after the study is started. This decision will not affect your right to receive the care you would receive if you were not in the study.
- 9) To receive a copy of the signed and dated consent form.
- 10) To be free of pressure when considering whether you wish to agree to be in the study.

If you have other questions, please ask the researcher or research assistant. In addition, you may contact the Office of Human Research Protection (OHRP), which is concerned with protecting volunteers in research projects. You may reach OHRP by calling (916) 734-6864, from 8:00 a.m. to 5:00 p.m., Monday through Friday, or by writing to the Office of Human Research Protection, Ambulatory Care Center - Ellison Building, UCDCMC, 4860 Y Street, Suite 3870, Sacramento, California 95817.

Signature of Participant

Date

Initials of Participant

Version #1, 2/11/2004

DESCRIPTION OF STUDY
UCDCC Study #141

Page 7 of 7

Alan Diamond, Ph.D. Dr. Diamond will provide nutritional advice to the project, as needed.

Cathy Hollister and Cheri Koppe, Clinical Research Associates. Ms. Hollister and Ms. Koppe will assist with coordination of the project, as needed (the Clinical Research Nurse will have primary responsibility for this).

In addition, investigators have the invaluable resource of the U.C. Davis Cancer Center, where the study is being conducted.

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UNIVERSITY OF CALIFORNIA, DAVIS

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SANTA BARBARA • SANTA CRUZ

OFFICE OF THE VICE CHANCELLOR FOR RESEARCH
Office of Human Research Protection
Telephone: (916) 734-6864
FAX: (916) 734-6872

SACRAMENTO, CALIFORNIA 95817

April 1, 2003

Dear Research Participant:

One of the major responsibilities of the UC Davis "Office of Human Research Protection" is to ensure that individuals contemplating participation in any research conducted by a member of UC Davis are fully informed of their rights under the federal law/regulations, and University policy. These laws/regulations/policies specifically address a research participant's rights to be fully informed of certain issues in regards to their participation in any research, as well as ensuring that the participation is voluntary and not coercive.

If you are considering enrolling in a research study, or are now enrolled in a study, I would like to encourage you to become familiar with your rights. Our web site has been designed to provide this important information to you. The following areas should be of major importance to:

- Your basic rights as a research subject.
- An explanation of the informed consent process, an essential step before you can be enrolled in any study.
- A basic requirement that the written consent be in a language understandable by you.
- Suggested sample questions to ask the research investigator regarding your participation in the study.

The section of our web site providing this information can be reached as follows:

<http://ovcr.ucdavis.edu/humansubjects/HSParticipants/HSParticipants.html>

Please note that our web site contains additional information, such as issues regarding the UC Davis Institutional Review Board, a committee which is charged with reviewing and approving the study involving human subjects, prior to initiating any research at UC Davis, or recruiting potential participants such as yourself.

I hope you will find this information helpful in deciding whether you want to participate, or continue your participation, in any research at UC Davis. Should you have any questions please feel free to contact our office by phone (916) 734-6864 from 8 am to 5 PM, Monday through Friday, or by writing to the following address:

University of California, Davis
Office of Human Research Protection
Ambulatory Care Center, Suite 3870
4860 Y Street
Sacramento, CA 95817

Thank you for your interest and participation in research at UC Davis.

Sincerely,

Lynne U. Chronister
Associate Vice Chancellor for Research Administration
Acting Director, Office of Human Research Protection
University of California, Davis

Initials of Participant

Version #1, 2/11/2004

Authorization for Release of Personal Health Information and Use of Personally Unidentified Study Data for Research**Study Title (or IRB Number if study title may breach subject's privacy):**

A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

Sponsor/Funding Agency:Department of Defense**A. Introduction:**

The federal privacy law called the Health Insurance Portability and Accountability Act (HIPAA) requires you to give your permission to release your Personal Health Information to the research team and others so that you can participate in this study. This form describes the different ways that the researcher, research team and the research sponsor may use your Personal Health Information for the research study. You must sign this form to participate in the study, but not signing won't have any other effect on your health care or insurance coverage.

B. Release of Personal Health Information

If you agree to participate in this research study and sign this form, you are authorizing U.C. Davis Cancer Center to release the following Personal Health Information. Your Personal Health Information contains specific health information about you and information that identifies you. For example, Personal Health Information may include your name, address, phone number and social security number.

C. Description of Your Health Information to be Released (check one or more):

- | | | |
|---|---|---|
| <input checked="" type="checkbox"/> Entire Medical Record | <input checked="" type="checkbox"/> Laboratory Reports | <input checked="" type="checkbox"/> Emergency Medicine Center Reports |
| <input checked="" type="checkbox"/> Health Care Billing Statements | <input type="checkbox"/> Dental Records | <input checked="" type="checkbox"/> History & Physical Exams |
| <input checked="" type="checkbox"/> Pathology Reports | <input checked="" type="checkbox"/> Operative Reports | <input checked="" type="checkbox"/> Diagnostic Imaging Reports |
| <input checked="" type="checkbox"/> EKG | <input checked="" type="checkbox"/> Radiology Reports | <input checked="" type="checkbox"/> Consultations |
| <input checked="" type="checkbox"/> Progress Notes | <input checked="" type="checkbox"/> Radiologic & MR Scans | <input checked="" type="checkbox"/> Outpatient Clinic Records |
| | <input checked="" type="checkbox"/> Discharge Summary | <input type="checkbox"/> Psychological Tests |
| <input checked="" type="checkbox"/> Other <u>Mental Health Diagnosis or Treatment</u> | | |

Initials of Participant

D. U.C. Davis Cancer Center May Release Personal Health Information to These People or Organizations for the Following Purposes:

1. To the researcher and members of the research team for the research purposes described in the attached Informed Consent Form, and to other individuals at UC who oversee the research, including the human research ethics review board;
2. To the Food and Drug Administration (FDA), to the research sponsor or the sponsor's representatives, or to other government agencies in the U.S. and other countries, as required by law to monitor the quality, safety or effectiveness of the study.
3. The research team will protect your information as described in the attached Informed Consent Form and will comply with the requirements of all applicable laws that protect the confidentiality of your Personal Health Information. Once your Personal Health Information is released, it may be redisclosed and not protected by HIPAA. Your Personal Health Information may be protected under other state or federal privacy laws.

E. Specific Authorizations

The following information will not be released unless you put **your initials** on the specific line(s).

- _____ I specifically authorize the release of information pertaining to drug and alcohol abuse, diagnosis or treatment (42 C.F.R. §§2.34 and 2.35).
- _____ I specifically authorize the release of HIV/AIDS testing information (California Health and Safety Code §120980(g)).
- _____ I specifically authorize the release of information pertaining to mental health diagnosis or treatment (California Welfare and Institutions Code §§5328, *et seq.*) as follows: _____
- _____ I specifically authorize the release of genetic testing information (California Health and Safety Code §124980(j)).

F. Use and Release of Personally Unidentified Study Data

If you agree to participate in this research study, the research team, the research sponsor and the sponsor's representatives may use Personally Unidentified Study Data. The Personally Unidentified Study Data does **not** include your name, address, telephone or social security number. Instead, the researcher assigns a code to the Personally Unidentified Study Data. Personally Unidentified Study Data may include your date of birth, initials and dates you received medical care. Personally Unidentified Study Data may also include the health information used, created or collected in the research study. The research team or the research sponsor may share the Personally Unidentified Study Data with others in the following ways:

1. To perform additional research, place it into research databases, share it with researchers in the U.S. or other countries, use it to improve the design of future studies, and publish it in scientific journals; or
2. To share it with business partners of the sponsor and to file applications with U.S. or foreign government agencies to get approval for new drugs or health care products.

G. Expiration

This authorization ends when the study ends and when all required study monitoring is over. The use of the Personally Unidentified Study Data has no expiration date.

H. Revoking Authorization

You can cancel ("revoke") this Authorization at any time. To cancel this Authorization, write to the researcher identified in the attached Informed Consent Form, or ask a member of the research team to give you a form to revoke the Authorization. If you cancel this Authorization, you may not be able to continue to participate in the research study. Also, you may not be eligible for medical treatment related to the research study. You may want to discuss with the research team the effect on your medical treatment of canceling this Authorization. If you cancel the Authorization, information that was collected about you may continue to be used. Also, the sponsor and government agencies may continue to see your medical records to monitor the research that was done before you cancelled the Authorization.

I. Authorization

By signing this Authorization you agree that you have been given the opportunity to ask questions and you agree to the release of your Personal Health Information and to the use and release of the Personally Unidentified Study Data as described in this form. If you have questions, you may contact the researcher. You will be given a signed copy of this Authorization. The above information supersedes any information contained in the consent form with regard to privacy protections. If you refuse to sign this, you may not receive research-related treatment. However, payment, enrollment or eligibility for benefits, and any treatment unrelated to research will not be affected by your refusal.

Subject's Name (print)

Subject's Signature

Date

For Minor Subjects or For Adults Incapable Of Giving Consent (where IRB approved):

Legally Authorized Representative's Name (print)

Relationship to the Subject

Representative's Signature

Date

Initials of Participant

Version #1, 2/11/2004

INFORMED CONSENT FORM

TITLE OF STUDY: DOD A-11241: A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study

PRINCIPAL INVESTIGATOR: Srinivasan Vijayakumar, M.D., Department of Radiation Oncology, UCDMC

CO-INVESTIGATORS: Samir Narayan, M.D., Janice K. Ryu, M.D.
Dept. of Radiation Oncology, UCDMC

CO-INVESTIGATOR: Ralph W. deVere White, M.D.
Department of Urology, UCDMC

CO-INVESTIGATOR: Paul Gumerlock, M.D.
Hematology & Oncology, UCDMC

CO-INVESTIGATOR: Laurel Beckett, Ph.D.
Epidemiology & Preventive Med., UCDMC

CO-INVESTIGATOR: Ralph Green, M.D.
Pathology, UCDMC

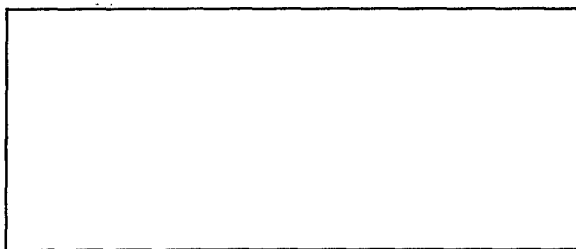
CO-INVESTIGATOR: William C. Baker, Jr., M.D.
Urology, VA Mather

CO-INVESTIGATORS Rajendra G. Mehta, Ph.D., Rajeshwari R. Mehta, Ph.D.; Surgical Oncology,
University of Illinois at Chicago [UIC]

CO-INVESTIGATOR Alan Diamond, Ph.D.
Human Nutrition, UIC

TELEPHONE NUMBER: (916) 734-7888 — Rad. Oncology, UCDMC

EMERGENCY NUMBER: (916) 734-2011



Initials of Participant

Consent to Participate in a Research Study
University of California, Davis

This is a clinical trial (a type of research study). Clinical trials include only patients who choose to take part. Please take your time to make your decision. Discuss it with your family and friends.

WHY IS THIS STUDY BEING DONE?

1.0 Why am I being asked to participate in this Study?

You have been diagnosed with prostate cancer. You also received radiotherapy for your prostate cancer. You may or may not know that the prostate cancer that was diagnosed in you had certain features that make you at a higher risk for the cancer to return--also called 'recurrence' --than others who may not have those factors in them.

1.1 What are those features that make me at a higher risk for cancer's recurrence?

Three known features make some prostate cancer patients at a higher risk for recurrence: Cancer Stage, Cancer Grade, and PSA levels.

- **Stage:** Stage is based on your doctor's digital rectal examination [DRE] prior to any treatment. It tells how much of your prostate gland is affected with cancer that can be felt by the doctor. Patients have stages T1 or T2 or T3 or T4. T1 and T2 are early; T3 and T4 are relatively more advanced stages.
- **Grade:** Grade is determined from the biopsy that was taken at the time of diagnosis of your prostate cancer, by looking through a microscope. This score goes from 1 to 10. A score of 6 or less is considered favorable. More than 6 is not. Sometimes these scores are also called "Gleason Sums."
- **PSA:** PSA or Prostate Specific Antigen is a blood test. PSA is secreted only by the prostate gland. All men have PSA in their blood; just having PSA in the blood is not evidence of having cancer. However, when these levels are higher than normal, one has to be concerned. The normal range in men without prostate cancer is **less than 4** nanograms/ml. A value higher than 10 nanograms/ml in men with prostate cancer is considered not so favorable.
- **Combining These Three Factors:** Studies by doctors and other scientists show that, by combining the three factors listed above, one can estimate the chances of the cancer's recurrence. If a patient has two or three of the above unfavorable factors, he has a higher chance of the prostate cancer's recurrence. For example: a patient whose stage is T3 or T4 with a Grade Score of 8 and / or PSA of 15 will be considered to have higher chances of cancer recurring than another man with a T1 cancer, Grade Score of 5 and PSA number of 5.

Initials of Participant

1.2 How can you tell that the cancer is recurring?

Blood PSA test is very useful in this. Generally, PSA numbers go down during the months following radiation therapy. Sometimes it takes a year or longer for PSA to reach the lowest level. There is no PSA level that may tell the doctors that the cancer may not come back, although lower than 4 is good; and the closer it is to zero, the better. It very rarely goes to zero after radiotherapy treatments, because the prostate gland is still left in place, unlike after surgery.

Your doctors usually want the PSA levels to be stable: that is, not increase over time when they see you after treatments – usually once every 4 months. A slight variation (within 1 nanogram/ml) is considered normal. Only when the values keep increasing steadily, is it considered an indication of the cancer coming back.

1.3 Do I have to sign-up for this study? What if I say 'No'?

This is a research study. So, your participation is completely voluntary. Your decision whether or not to participate will not affect your current or future relations with your doctors or other health care providers in the University. Furthermore, if you decide to participate, you are free to withdraw at any time without affecting that relationship.

2.0 Why is this research being done?

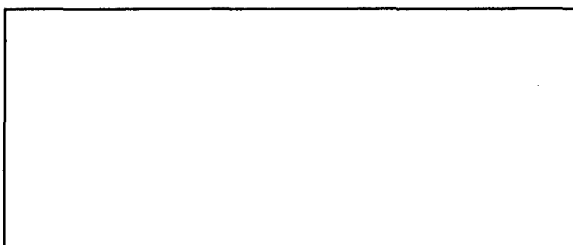
Currently, although there are treatments available for prostate cancer recurrence, they often have many side effects and complications, such as loss of sexual desire, inability to get penile erections, loss of calcium from the bones, etc. Often, these treatments have to be continued life-long. So doctors and scientists are trying to develop medicines that can try to prevent or delay the cancer recurrence. That way, the complications of treatment at the time of cancer recurrence can be avoided. Also, the treatments when cancer recurs are expensive; by preventing such recurrence, health care expenses can be reduced.

3.0 What is the purpose of this research?

The purpose of this research is to see whether a medicine called 'Vitamin D5' – a modified form of Vitamin D – can be taken safely without significant side effects by prostate cancer patients who had received radiotherapy. Another purpose for the research is to see whether taking Vitamin D5 daily for two years (10 µg per day) will decrease the chances of cancer recurrence.

4.0 What is vitamin D5 and how is it different from 'regular' vitamin D?

Scientists at the University of Illinois at Chicago, colleagues of this study's investigators, have modified the chemical structure of 'Regular' Vitamin D to make a new compound called Vitamin D5 [Study Medication].



Initials of Participant

4.1 Vitamin D Patient Handout

Please refer to the handout for general information on Vitamin D from the National Institute of Health's (NIH) website.

4.2. How about vitamin D5? Why may this be better than just taking 'regular' vitamin D?

Vitamin D is important in the life cycle of the cell. Vitamin D affects the body because it changes to other related compounds – often called analogs. One analog, or equivalent, of vitamin D is D3, which holds back cell creation of many cell types, including prostate cancer. However, the use of D3 with patients is limited because it causes bad side effects at a strength required to stop cancer cell growth. Therefore, numerous other equivalents of vitamin D have been developed and evaluated for whether they might suppress the growth of cancer and whether they cause side effects. D5 is one of the analogs of vitamin D developed by the scientists at the University of Illinois at Chicago. In fact, of several hundred analogs, D5 is one of only a few that have been successfully used in animals at doses that do not cause very bad side effects.

4.3. What have the scientists at the University of Illinois at Chicago shown about Vitamin D5?

- They have shown:
 - Vitamin D5 is believed to cause the least amount of bad side effects of a series of vitamin D equivalents (vitamin D2 to vitamin D7).
 - Vitamin D5 can be tolerated at higher doses than many of the other analogs of vitamin D.
 - It inhibits cell transformation [that is a preliminary step towards cancer formation].
 - It slows the growth of breast cancer cells.
 - It also slows the growth of LNCaP cells – an experimental type of prostate cancer cells - in laboratory experiments and in experiments in mice.

4.4. What do the above findings mean to prostate cancer patients?

Vitamin D5 may slow down or delay the growth of prostate cancer. It is worthwhile studying whether Vitamin D5 is effective in treating men with prostate cancer. That is why you are being asked to consider participating in this research study.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

There will be 40 patients in this study.

Initials of Participant

Version #1, 2/13/2004

WHAT IS INVOLVED IN THIS STUDY?

5.0 If I decide to participate, what procedures are involved? What is expected of me?

5.1 Checking Your Eligibility

To qualify to participate, you have to meet certain criteria that are detailed in the Protocol given to you with this Consent. Your doctor will check all the criteria and may also discuss with your primary doctor and Urologist before registering you for the study. You also have to sign this Informed Consent form and initial every page after carefully reading it, asking all the questions you want to ask, and satisfying yourself that those questions were answered fully.

5.2 Trial 'Run-in' Period

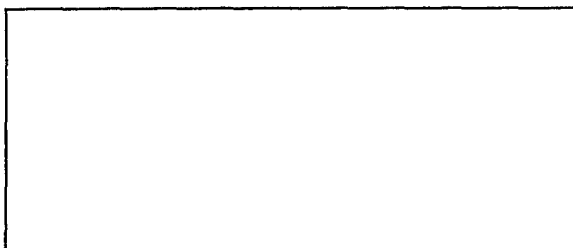
Before you begin the study, you will take part in a trial run-in period. This lasts one month, during which you will be required to take the placebo medication (sugar pill)--one pill a day. This is to ensure that you are able to remember to take one pill every day regularly. At the end of this period, you have to bring back the bottle of pills that was given to you and the Pill Calendar form that you have been filling out. We will look at the calendar and count the number of pills that remain in the bottle to determine your eligibility. This has to be within 10% of the expected count (no more than 3 pills missed). We do depend on your honesty to tell us whether you are able to comply well. If not, you should inform us and we will not proceed to include you in the study. You can voluntarily withdraw from the study at this time.

5.3 Randomization

Once you have signed the consent form, you will be placed in one of two groups for the study (20 patients in each group). This is called being "randomized" and is like tossing a coin and being placed in a group based on how the coin lands. You will be assigned to either the "study arm", where you will receive the study medication (10 µg per day) or to the "placebo arm", where you will receive the so-called "sugar pills". However, neither you nor your doctors or the health care professionals who help you with the study will know which kind of pills you are receiving. Only the statistician will know. This ensures unbiased conduct of the study. [However, under certain circumstances, for example life-threatening illnesses, the process can be 'unblinded'; that is, you and your doctor will be informed of which pills you were taking.]

5.4 Taking the Pills

You have to take one pill (10 µg per day) every day for two years. This is the main part of the study. There is no particular time of day that you must take the pill; however, it is



Initials of Participant

better if you take it at the same time every day so that you do not forget to take a pill, or take more than one pill a day. If you forget on a particular day, please do not take two pills the next day. In other words, do not try to make up for the days you forget to take the pill. Just resume taking one pill a day and inform us about the number of days you forgot to take a pill when we call you or when you see us during the next follow-up visit. Record your pill use on the calendar provided.

5.5. Follow-up

This consists of two phases: an Intensive Initial Follow-up Phase and a second 'Regular' Follow-up Phase.

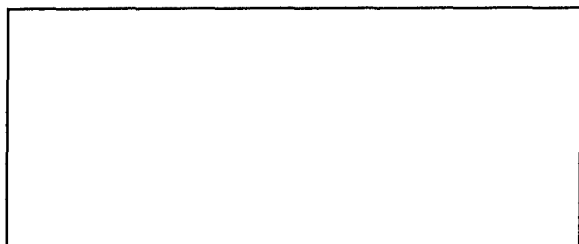
5.5.1 Intensive Follow-up

The follow-up with you will be intense, to detect any side effects as soon as possible, if they occur. That way, we can decrease the dose of the medication without delay.

For the first month, we will see you approximately once a week. Our Clinical Research Associate will interview you for any side effects you might experience. They will also draw blood [about 13 cc's, which is about three tablespoons] for tests [blood calcium levels and other tests]. If any side effects are reported by you or found in the blood test, then the dose of the medication can be modified. Our doctors may also interview you and examine you.

If everything goes well for a month, then the visits will be required only once a month for the next three months. However, our Clinical Research Associate will telephone you about once a week to confirm that you are doing all right. When you visit us once a month, the procedures will be the same as the once-a-week visits in the first month.

At the end of the four months, you will have a 'regular' follow-up, meaning, your radiation oncology doctor will also examine you and you will fill in some forms. Blood will be drawn for PSA, calcium, and other tests. For the next four months [that is, until 8 months into the study, starting from the first day you began taking the pills], our Clinical Research Associate will telephone you about once a month. In between the telephone calls, if you notice anything unusual with your health or experience new or unexplained symptoms, please call us at 916-734-3604. If in doubt, please call us. This will help you and us to properly conduct the study.



Initials of Participant

5.5.2. Regular Follow-up Visits

After the Initial Intensive Follow-up, you will be seen once in four months, earlier if necessary. These visits will be exactly the same as the first 4-month visit, which included a physical examination, a digital rectal examination, blood tests [about 13 cc's, which is about 3 tablespoons, drawn from your vein] and filling in some forms. These forms will ask questions about your quality of life; that is, whether there are any changes in your abilities or enjoyment. These forms are several pages long. These are the same forms you were asked to complete at the beginning of the study. Our Clinical Research Associate will help you to complete them if you have any questions.

5.5.3 End of Study Visit and Biopsy

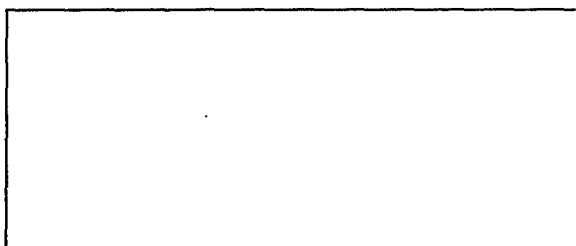
At the end of two years, you will have a final visit similar to your other follow-up visits. This will be followed by an Ultrasound Guided Biopsy of your prostate – this is similar to the biopsy procedure you had prior to your radiotherapy treatments. This will be done by the Urologist – either your own Urologist or the UCD Urologist. Tests will be done on the biopsied tissue. These include tests to see whether the cancer has recurred and other tests to see whether the pills you have been taking have made any changes in the cancer process. Cancer processes are the changes that precede or accompany conversion of normal cells to cancer cells. However, many of these processes continue to be discovered.

5.5.4 Keeping in Touch

You will be informed of the test results. After all patients have completed their biopsies and once we have analyzed the findings, these results will also be told to you. You are requested to continue to see your radiation oncologist once every 4 months for the next three years and less frequently afterwards. You will be notified of any new information. You will be informed of the results of our research in a letter sent to you after we have completed our final analysis.

HOW LONG WILL I BE IN THE STUDY?

You will be in the study for two years. Please review the chart below for a summary of what will be required, and when it will be required.



Initials of Participant

Version #1, 2/13/2004

Summary of Schedule for Study Participants									
Month(s):	Run-in		1		2-4		5, 9, 13, 17, 21		24
Event/Procedure	Week(s):		1	2-5	1	2-5	1	2-5	5
Informed Consent Given	X								
Clinic Visit	X	X	X	X	X		X		X
Physical exam by doctor	X						X		X
Digital Rectal Exam (DRE)	X						X		X
Complete History	X								
Lab Collection ¹	X	X					X		X
Clinical Research Associate (CRA) Interview	X	X	X	X	X		X		X
Quality of Life forms completed	X		X		X		X		X
AUA GU Symptom Scoring Scale completed	X				X		X		X
Karnofsky Performance Scale completed	X	X	X		X		X		X
Pill Calendar given	X		X		X		X		X
Placebo pills given	X								
Placebo pills taken daily	X	X							
Pill Calendar collected			X		X		X		X
Study pills given (10 µg per day)			X		X		X		X
Study pills taken daily			X	X	X	X	X		
Patient symptoms documented			X		X		X		X
PTH			X				X		
Biopsy (following initial biopsy at time of diagnosis)									X
Telephone Call by CRA				X	X	X			

¹ for PSA, calcium, phosphorus, albumin, Chem 7, and urine electrolytes

WHAT ARE THE RISKS OF THE STUDY?

6.0 What are the potential risks and discomforts?

There are a number of potential risks and discomforts that you should be aware of before you consent to participate. You will be informed of any significant new findings developed during the course of the research that could affect your willingness to continue participation. The investigational agent to be used in this study is not approved by the Food and Drug Administration (FDA) for commercial use; however, FDA has permitted its use in this research study.

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6.1 Potential Side Effects and Complications from the Study Medications

Although preliminary studies have indicated a relative safety of the Study Medication, one of the purposes of this study is to see whether there are any unexpected side effects from it. One of the known side effects of Vitamin D, when taken in excess or when the potent analogs are used, is an increase in blood calcium levels – this is called "Hypercalcemia". The symptoms of Hypercalcemia are listed in the tables below.

Some Potential Risks Associated with the Study Procedures		
<i>Procedures</i>	<i>Risks</i>	<i>Measures to Minimize Risks</i>
Taking the drug 1 α (OH)D5 for 2 years	Hypercalcemia: symptoms include loss of appetite, nausea, vomiting, abdominal pain, constipation, and other symptoms (see 2 nd table below). There may also be unknown effects since the study drug is a newly synthesized analog of vitamin D.	During the 2-year treatment period, subjects will be examined weekly, monthly, and then every 4 months. They also will be telephoned by the Clinical Research Associate weekly or monthly about the side effects they are experiencing, and the dosage of study drug will be adjusted or will be stopped temporarily or permanently as necessary.
Providing blood samples weekly, monthly, and every four months (2-3 tbsp each), over the course of two years	1) Pain, local bruising, bleeding, possible infection. 2) Possible breach of confidentiality.	1) Blood collection methods used in the study are the same as those used for routine clinical exams. 2) Procedures have been established for confidential collection, labeling, storage, use, and disposal of blood samples.
Ultrasound guided biopsy of prostate at the end of two years	Since needle biopsy will be used, risks are discomfort, local bleeding, small bruise, tenderness, infection (rare), and allergic reaction to local anesthesia.	
Completing "Quality of Life" survey several times during 2-year period	Inconvenience of completing forms	

Initials of Participant

<i>Procedures</i>	<i>Risks</i>	<i>Measures to Minimize Risks</i>
Telephone interviews by Clinical Research Associate done weekly or monthly during 2-year period	Inconvenience of completing interviews	
Physical exam and digital rectal exam by radiation oncologist several times during 2-year period	Minor discomfort	

Symptoms of Hypercalcemia can be:

- Loss of appetite, nausea, vomiting, abdominal pain, constipation, inflammation of pancreas, stomach or intestinal ulcers
- Confusion, memory loss, tiredness, depression, even fainting
- Excessive urination, more frequent urination, including at night, kidney stone formation
- Muscle weakness, muscle aches, bone pain
- Increase in blood pressure, calcium deposits in the soft tissues of the body, a band formation in the cornea of the eye
- Itching
- HOWEVER, MOST PATIENTS DO NOT HAVE ANY SYMPTOMS. THAT IS ONE OF THE REASONS WE HAVE DESIGNED THIS STUDY WITH A PERIOD OF INTENSIVE FOLLOW-UP IN THE INITIAL FOUR MONTHS: TO IDENTIFY ANY OF THESE SYMPTOMS EARLY AND INTERVENE IF NECESSARY.
- ALSO, DEVELOPING SYMPTOMS DEPENDS UPON HOW LONG AND HOW RAPIDLY CALCIUM LEVELS INCREASE IN THE BLOOD. THE SHORTER THE DURATION AND LESS RAPID THE INCREASE, THE LESS ARE THE CHANCES OF DEVELOPING SIDE EFFECTS. THAT IS WHY, AGAIN, WE HAVE DESIGNED THE INTENSIVE FOLLOW-UP PERIOD TO DETECT ANY HYPERCALCEMIA AS SOON AS POSSIBLE, IF IT OCCURS.
- There may be other unknown and unexpected complications that could occur, including life-threatening complications.

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Version #1, 2/13/2004

6.2 Blood Drawing

The most frequent risks are bruising, pain at the site of needle stick, bleeding, and infection. The amount of blood drawn is unlikely to lead to anemia (low blood cell count).

6.3 Follow-up visits and completion of forms

Generally, prostate cancer patients are seen every four months after they complete radiotherapy, undergo a doctor's examination (including a digital rectal examination), and get blood drawn at the time of follow-up visits for PSA. So the follow-up schedule for the study is not any different than in other patients except during the initial phases. In addition, the number of telephone calls and the necessity of completing many forms can be inconvenient and may interfere with your routine life.

6.4 Biopsy

This has the same risks and discomforts as the biopsy you had at the time of your diagnosis: A needle biopsy can be painful. Risks include bleeding and infection. You may notice blood in your urine, in your semen, or with a bowel movement for several weeks after the biopsy.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?**7.0 What are the benefits in taking part in this study?**

You may have no benefit by participating in the study. You will, however, help other patients if Vitamin D5 is found to be an effective drug in controlling prostate cancer. If Vitamin D5 has beneficial effects in controlling prostate cancer, then there is about a 50% chance of your being in the study arm, thus receiving the Vitamin D5 medication and any benefits it may have (it may have none).

WHAT OTHER OPTIONS ARE THERE?**8.0 What other options are there for me?**

Since there is no evidence at this time that your cancer has spread, you do not have to take any other medications. Your doctors can follow you periodically at regular intervals.



Initials of Participant

9.0 Will I be told about new information about me that may affect my continuing in the study?

Yes, you will be. For example, if your cancer spreads during the two-year study period, you will be informed and will be taken off the study. Or if you develop any unexpected side effects, you will be informed and will be taken off the study.

WILL ANY BIOLOGICAL SAMPLE(S) BE STORED AND USED IN THE FUTURE?

Part of this study will include testing a biopsy of your prostate. We would like to keep some of the tissue for future research. This tissue will be stored and may be used in research to learn more about cancer and other diseases. You have a say in how your stored samples are used in the future research. Reports about the research done with your tissue and/or blood will not be given to you or your doctor because the research will not have an effect on your care. **Please read the attached "Tissue Sample Consent Form". If you choose not to allow your tissues to be kept for future research, this will not affect your cancer care in any way.**

WHAT ABOUT CONFIDENTIALITY?**10.0 What about privacy and confidentiality?**

The only people who will know that you are a research subject are members of the research team, members of the UCD Institutional Review Board, and, if appropriate, your physicians and nurses. No information about you, or information provided by you during the research, will be disclosed to others without your written permission, except:

- if necessary to protect your rights or welfare (for example, if you are injured and need emergency care); or
- if required by law.

Representatives of the U.S. Army Medical Research and Materiel Command (USAMRMC) are eligible to review research records as a part of their responsibility to protect human subjects in research.

When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity.

Authorized representatives of the Food and Drug Administration (FDA) may need to review records of individual subjects. As a result, they may see your name; but they are bound by rules of confidentiality not to reveal your identity to others.

Initials of Participant

To prevent access by unauthorized personnel, personal information, research data, and related records will be coded, stored, etc. in the following manner.

The data will be kept in a secure place within the Departmental vaults and/or computer, where access will only be possible only with appropriate passwords and available only to the PI and personnel involved in conducting the studies.

You consent to allow the FDA and/or a funding agency, such as the U.S. Army Medical Research and Materiel Command, access to your medical records should they deem it necessary.

DISCLOSURE OF PERSONAL AND FINANCIAL INTEREST IN THE RESEARCH STUDY AND SPONSOR:

Samples taken during this study may be used for research and development purposes not related to your treatment or condition. You will not have any property rights or ownership interest in products or data which may be derived from your samples.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

11.0 What are my rights as a research subject?

If you have questions about your rights as a research subject, you may call the U.C. Davis Office of Human Research Protection at (916) 734-6860.

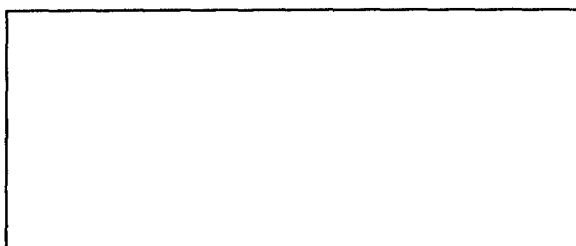
12.0 Can I withdraw or be removed from the study?

As indicated earlier, your participation is voluntary. You can choose to withdraw at any time from the study. This will not affect your relationship with your doctors or UCD. The details on this have been mentioned earlier. The circumstances in which you may be removed from the study have also been detailed before; one additional reason can be any other physical or mental illness that may interfere with your successful participation in the study as determined by your doctors.

13.0 What if I am injured as a result of participation?

All forms of medical diagnosis, treatment, and research, whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop complications from participation in this study.

There is no rescue medication for this study. If you experience adverse effects from the study medication (D5) you will stop taking D5 and be provided necessary clinical support. Most research-related injuries will be treated and resolved by UC Davis Medical



Initials of Participant

Center, which will follow its usual policy for emergency care. There is no compensation and/or payment for such medical treatment from the UCD Medical Center for such injury except as may be required of the University by law.

If you are hurt or get sick because of this research study, you can also receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic. In the event you need non-emergency care, the Principal Investigator, Dr. Vijayakumar, will call the Army if you have a research-related injury that UC Davis is unwilling to treat, or if you for some reason want to explore Army treatment (at an Army Medical Treatment Facility[MTF]) even though UC Davis has offered treatment.

The nearest Army MTF is located at Fort Irwin, California, at the Weed Army Community Hospital. Dr. Vijayakumar cannot promise medical care from that Army MTF as he is not the one who will determine your eligibility. If the Army finds you eligible for Army MTF care (because the Army agrees that the injury is research-related), then it is possible you can get medical care at an Army MTF. However, you should not call the Army MTF directly, because that is not how eligibility will be determined.

If you have questions about this medical care, talk to the principal investigator for this study, Dr. Srinivasan Vijayakumar, at (916) 734-7888. If you pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

If you feel you have been injured, you may contact:

- Dr. Vijayakumar, Principal Investigator, at (916) 734-7888
- Dr. Narayan at (916) 734-8051
- Dr. Ryu at (916) 734-8251
- Any of our Clinical Research Associates:
 - Clinical Research Nurse (to be named)
 - Cheri Koppe at (916) 734-3604
 - Cathy Hollister at (916) 734-8814

Initials of Participant

COMPENSATION / COSTS**14.0 What are the costs for participating in the Study?**

You will not be charged or paid to participate in the study. The study medications will be provided to you free of cost. The routine blood tests that are part of your regular follow-up will be paid by your insurance company or by you, as in the case of a patient who had received radiotherapy and was being followed by his doctors. You will not be charged for any of the special blood tests that are specifically designed for the study.

It is possible that your insurance will not pay for all of the treatments and tests you will receive if you participate in the research. That is because many insurance companies, HMOs, and health benefits plans do not cover experimental treatments. You give us permission to submit bills to any appropriate third parties (insurance carriers).

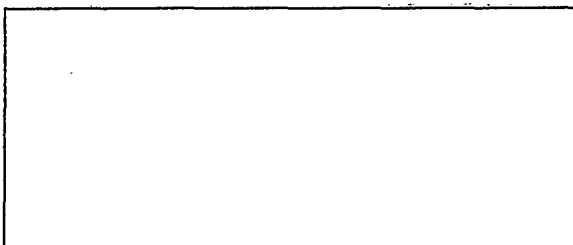
All routine diagnostic laboratory tests and follow-up office visit costs necessary for your treatment will be borne by your insurance company (i.e., HMO or other health benefit provider). However, if your insurance company refuses to reimburse you, then you will be billed for these procedures. There will be no charge for the drug(s) or some of the specific tests performed to gather scientific information regarding this form of vitamin D. The biopsy at the end of the study carries the same risks as the biopsy you had at the time of diagnosis, and will not cost you any additional expense.

As stated above, if you are hurt or get sick because of this research study, you can receive medical care at an Army hospital or clinic free of charge. You will only be treated for injuries that are directly caused by the research study. The Army will not pay for your transportation to and from the hospital or clinic.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?**15.0 Who should I contact if I have any questions?**

You can contact any one of the doctors listed in the first page of the consent form or any of the Clinical Research Associates listed on that page. Here are the specific numbers:

- Dr. Vijayakumar, Principal Investigator, at (916) 734-7888
- Dr. Narayan at (916) 734-8051
- Dr. Ryu at (916) 734-8251



Initials of Participant

Any one of our Clinical Research Associates:

- Cheri Koppe at (916) 734-3604
- Cathy Hollister at (916) 734-8814

Also, if you have any questions regarding your rights and participation as a research subject, please contact the Office of Human Research Protection at (916) 734-6897 or write to the University of California, Davis, Office of Human Research Protection, Ambulatory Care Center, Suite 3870, 4860 Y Street, Sacramento, CA 95817.

WHERE CAN I GET MORE INFORMATION?

The following information is provided to you as sources of more general information about clinical trials, disease course and standard treatment for most types of cancer. You may call the NCI's Cancer Information Service at: 1-800-422-6237 or TTY at 1-800-332-8615.

Helpful Web sites:

- **cancerTrials:** comprehensive clinical trials information at <http://cancertrials.nci.nih.gov>
- **CancerNet:** accurate cancer information on most types of cancer (PDQ) at <http://cancernet.nci.nih.gov>

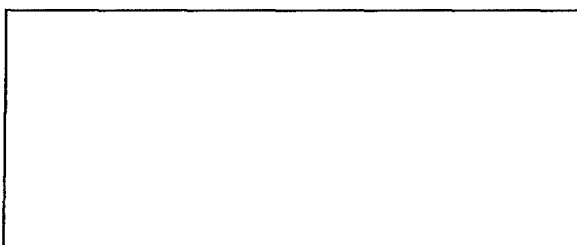
VOLUNTARY CONSENT

16.0 Statement of Voluntary Participation

Remember:

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future relations with the University of California, Davis or the U.S. Army Medical Research and Materiel Command. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

You will be given a copy of this form for your information and to keep for your records. You will also be given a copy of the study protocol and the Experimental Subjects Bill of Rights.



Initials of Participant

17.0 Statement of Consent and Dated Signature

Your signature below will indicate that you have decided to volunteer as a research subject, and that you have read and understood the information provided above, and the Bill of Rights.

Signature of Subject or Legally Authorized Representative

I have read (or someone has read to me) the above information. I have been given an opportunity to ask questions and my questions have been answered to my satisfaction. I have been given a copy of this form.

Signature of Participant

Date

Time

Printed Name

Signature of Physician

Date

Time

Please send this Consent Form to either one of this study's Clinical Research Associates:

*Cherri Koppe or Cathy Hollister
Clinical Trials, Radiation Oncology
U.C. Davis Cancer Center
4501 X Street, Suite G-126
Sacramento, CA 95817*

Initials of Participant

Version #1, 2/13/2004

CLINICAL PROTOCOL

**A Phase I/II Double-Blinded, Randomized Clinical Trial
to Prevent/Delay Biochemical and Clinical Failure
in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy,
Using 1 α -Hydroxyvitamin D5 Versus Placebo:
A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study**

University of California, Davis Medical Center (UCDMC)
and

University of Illinois at Chicago (UIC)

A Department of Defense-Funded Study

(Award No. DAMD17-02-1-0070, HSRRB Log No. A-11241)

Principal Investigator:

Srinivasan Vijayakumar, M.D.

Radiation Oncology, UCDMC

Co-Investigators:

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Alan Diamond, Ph.D.

Human Nutrition, UIC

Clinical Research Associates

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Radiation Oncology, UCDMC

Cheri Koppe

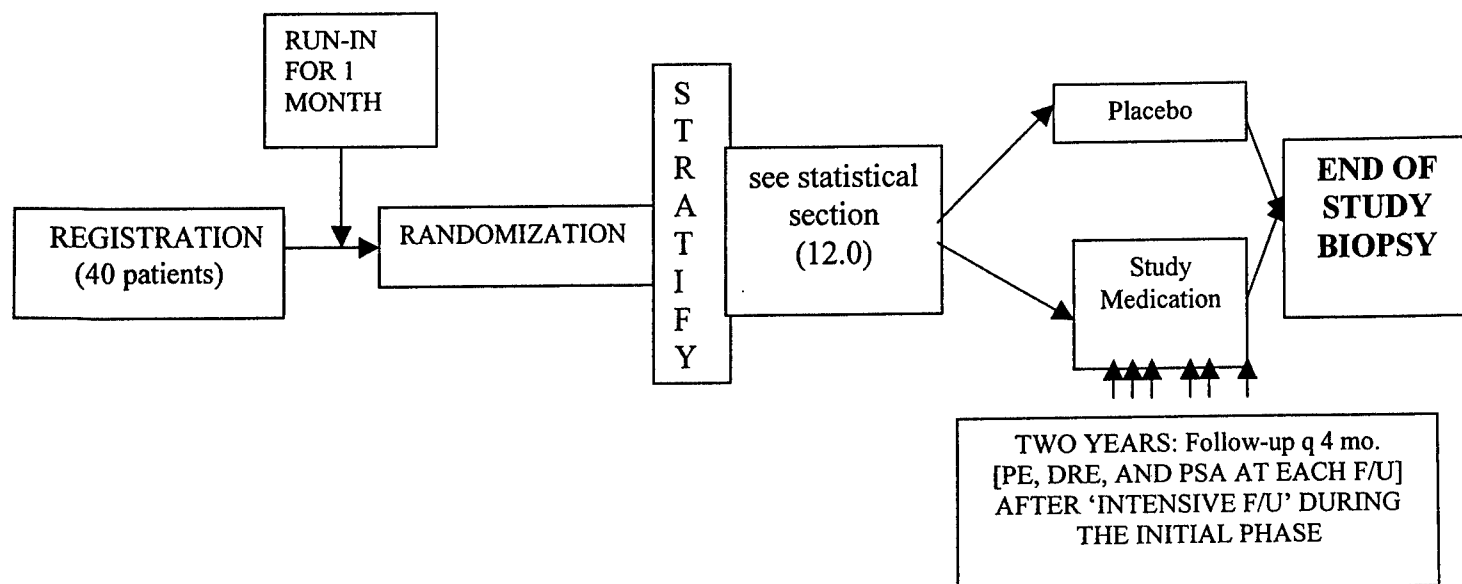
Radiation Oncology, UCDMC

Clinical Research Nurse (To Be Hired)

Radiation Oncology, UCDMC

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STUDY SCHEME

1. PURPOSE, METHODS, AND PROCEDURES

PURPOSE

The prostate gland is left *in situ* after radiation therapy; hence, the phenomenon of "field changes" and the factors responsible for prostate cancer initiation, promotion, and progression continue to operate in the prostatic cells. This results in radiation therapy failure [PSA and/or clinical] in 30-50% of patients. We hypothesize that treating patients with a relatively non-toxic chemopreventive and therapeutic agent, 1 α -hydroxyvitamin D₃, post-radiation therapy will prevent or delay the local recurrence of prostate cancer in radiation-treated prostate cancer patients. This will enhance their outcome results, including the quality of life (QOL) in patients receiving radiation therapy (RT) as the primary modality. In this study we are targeting high and intermediate risk patients, who are more likely to be in the 30-50% of patients that will have radiation therapy failure (see inclusion and exclusion criteria).

BACKGROUND

NON-METASTATIC PROSTATE CANCER, ADVERSE PROGNOSTIC FACTORS, AND THE USE OF RADIOTHERAPY

Non-metastatic Prostate Cancer and the Prognostic Factors that Affect the Outcomes within that Stage

The American Cancer Society estimates that 220,900 new prostate cancers will be diagnosed in the year 2003 in the U.S. and 28,900 men will die of prostate cancer in 2003 [Jemal et al., 2003]. Prostate cancer constitutes 33 % of all cancers among men and 10 % of cancer-related deaths [Jemal et al., 2003]. Over 90 % of cases diagnosed between the years 1992 and 1997 were non-metastatic, representing a stage migration, influenced by prostate specific antigen [PSA]-based screening efforts [Vijayakumar et al., 1998; Jani et al., 2001]. Yet, many patients with localized prostate cancer carry adverse prognostic characteristics, and these patients carry higher chances of failure and development of metastases [Chuba et al., 2001] and death [Satariano et al., 1998]. For instance, in a National Patterns of Care [POC] study, a higher T-Stage, Gleason Sum [GS], and pre-treatment PSA levels predicted worse outcomes. In the Univariate analysis, cause-specific failure was significantly lower for higher T stage ($p = 0.014$), GS ($p = 0.001$), and pretreatment PSA ($p = 0.0004$); overall survival was significantly lower in patients with higher T stage ($p = 0.047$) or GS ($p = 0.0191$). This study had 600 patients treated in 71 institutions in the U.S. Other individual institutional data also suggest the prognostic importance of those three factors: T stage, GS and pre-treatment PSA [Zelevsky et al., 1998; Connel et al., 2001; Anderson et al., 1997]. Many researchers sub-stage these patients into three categories: Group I – Favorable; Group II – Intermediate Risk; Group III – High Risk [Zelevsky et al., 1998; Connel et al., 2001].

Options of Treatment for Non-metastatic Patients

Radiotherapy [RT] and radical prostatectomy [RP] are considered the treatments of choice for most patients with non-metastatic prostate cancer, with equal long-term outcomes [Abdalla et al., 2002]. Numbers Needed to Treat [NNT] calculations in a recent evidence-based study favors RT in terms of quantitative outcomes [Abdalla et al., 2002]. However, significant controversy exists as to the superiority of RT vs. RP, and a discussion on this issue is beyond the scope of this protocol. This study is for patients who have received RT.

The Extent of Biochemical Failure after RT

Between 30-50% of newly diagnosed non-metastatic prostate cancer patients undergo RT – either by their own choice or based on their physicians' recommendations [Savage et al., 1997; Yan et al., 2000; Shaw et al., 2000; Brandeis et al., 2000; Meltzer et al., 2001]. So, of the approximately 221,000 men diagnosed with prostate cancer, 90% [199,000] have non-metastatic cancer; of these, 66,300 [30%] to 110,500 [50%] will undergo radiotherapy. With a 30-50% rate of biochemical failure, as many as 33,000 to 55,000 patients will need an intervention that is now often a Total Androgen Blockade or Near-Total Androgen Blockade with LHRH-Agonist, which has a resultant loss of quality of life and significant cost.

Among these patients who undergo RT – either external beam or brachytherapy – between 30-40% will have PSA-based biochemical failure, mainly in those who had more than one or two advanced prognostic features among the three factors described above, viz, T3 or T4 stage, GS ≥ 6 , or PSA ≥ 10 ng/ml [Zelevsky et al., 1998; Connel et al., 2001; Anderson et al., 1997; D'Amico et al., 2000; Shipley et al., 1999]. Recent evidence indicates that initial PSA values and [PSA-based] biochemical failure predict future clinical failure and prostate cancer-related death [Jani et al., 1999; Kupelian et al., 2002; Small et al., 2001; Palmberg et al., 1999].

From the above discussion, the following can be concluded:

- A significant percentage of 221,000 newly diagnosed patients undergo RT as their primary modality of treatment.
- Among these, a significant proportion carry poor prognostic features – individually or in combination – such as T3-4 disease, Gleason Sum of ≥ 6 , and/or pretreatment PSA values of ≥ 10 ng/ml.
- These patients have higher chances of failure.
- The current intervention that is often used – the use of Androgen Blockade - significantly interferes with the quality of life and is quite expensive since these therapies are often continued for life and these patients have close to 85-90% 10-year survival rates [see Section 2.3 below].
- Post-treatment PSA levels can be used to detect early failures, and such PSA-based biochemical failures can be used to identify those patients who are likely to develop subsequent metastases.

THE MULTI-CENTRIC NATURE OF PROSTATE CANCER, "FIELD-CHANGES," AND PRE-MALIGNANT LESIONS

Prostate Cancer is a Multi-centric Disease

There is a general consensus that high-grade PIN lesions are precursors of subsequent development of prostate cancer [see for example: Sakr and Partin, 2001; Haggman, et al. 2000; Bostwick et al., 2000; Foster et al., 2000]. PIN is characterized by cellular proliferation within preexisting ducts and glands with cytological changes mimicking cancer. [Foster et al., 2000; Sakr et al., 2001; Qian J., 1998; Haggan et al., 1997].

The Reasons for Concluding that High-grade PIN Lesions are Likely Precursors of Subsequent Development of Prostate Cancer

- There is a significantly increased risk that patients with isolated high-grade PIN [HGPIN] will have prostate cancer confirmed on subsequent biopsy.
- HGPIN is found in association with cancer in 63% to 94% of malignant and 25% to 43% of benign prostates in autopsy studies.
- Data on age and race suggest that African-American men develop more extensive HGPIN at a younger age than white men. African-Americans have the highest incidence of prostate cancer in the world, about 1½ times higher than in U.S. whites. The mean age at diagnosis is also lower for African-Americans than for white Americans.
- A wide spectrum of molecular/genetic abnormalities appears to be common to both HGPIN and prostate cancer [for example: loss of 8p, 10q, 16q, 18q, and gain of 7q31, 8q, multiple copies of the c-myc genes, along with changes in chromatin texture, telomerase activity, etc.; Sakr and Partin, 2001; Foster et al., 2000].
- Cytogenetic links have been shown between high-grade pre-invasive neoplasia [PIN] lesions and prostate cancer [Alcaraz et al., 2001; Foster et al., 2000]. [For example: FISH analysis showing a high correlation (75% cases) in ploidy [aneuploidy] and pattern of cytogenetic alterations [trisomy 7, trisomy 8, and monosomy 8] between high-grade PIN areas and the paired prostate cancer focus in the same specimen [Alcaraz et al., 2001]. Similar findings are reported by Zitzelsberger et al. [2001].
- The incidence of PIN steadily increases with age of the general population, and African-American males have increased incidence of high-grade-PIN, which is highly correlated with increased incidence of prostate cancer [Powell et al., 2000].

The Causes of Failure Locally within the Prostate Gland after RT are not Well-established

There are two likely possibilities: (a) clonal growth of radio-resistant cells that survived the irradiation and/or (b) new development of malignant cells from normal or precancerous cells present in the prostate at the time of irradiation. It is not clear at this time what factors act upon the normal or precursor cells in the prostate in the process of malignant transformation; however, there is no reason to believe that whatever factors acted upon the prostate glandular cells in a patient prior to RT would change after RT. Thus, the prostate glandular cells left intact after RT are likely to become malignant once again. Consequently, chemopreventive agents that can stop

or delay the transformation process from normal and/or premalignant lesions to malignant lesions need to be studied.

TREATMENT FOR BIOCHEMICAL FAILURE AND THE DETERIORATION OF QUALITY OF LIFE

No standard treatment exists for the management of patients whose failure is detected based on PSA criteria. The current options include Androgen Ablation with LHRH-Agonists with or without Oral Anti-Androgens [Sylvester et al., 2001], Salvage RP for biopsy-documented local [prostate gland only] failure [Vaidya and Soloway, 2001], Intermittent Androgen Suppression [Crook et al., 1999], and observation alone.

All the interventions carry morbidities and losses of quality of life.

- Androgen Ablation is associated with hot flashes, loss of libido, inability to attain penile erection, tiredness, gynecomastia, and loss of bone mineral density.
- Intermittent Androgen Ablation carries the same complications as Androgen Ablation, except that, during the period when the patient is not receiving the LHRH-agonists, his side effects may subside.
- Salvage RP is rarely practiced and only a few Uro-Oncologists perform such procedures in a highly selected number of patients. A study conducted by CALGB in which the PI for the current study was a co-PI tested the feasibility of salvage prostatectomy. Fewer than five patients were accrued over three years. If performed, the chances of incontinence and impotence are higher than those associated with 'up-front RP' [i.e., those performed as first line of treatment at the time of diagnosis of prostate cancer].

For the above reasons, any intervention that can prevent or delay a biochemical failure is highly desirable.

VITAMIN D ANALOG - 1,25(OH)₂D₃ - AS A CHEMOPREVENTIVE AGENT

The role of vitamin D in cell proliferation and differentiation has been well established (Mehta and Mehta 2002, Miller 1999). Vitamin D and its analogs have shown laboratory and clinical evidence of chemoprevention and cytotoxic activity (Chen 2003; Guyton 2003; Krishnan 2003). The active metabolite of vitamin D 1 α , 25-dihydroxyvitamin D₃ suppresses cell proliferation of many cell types, including prostate cancer (Mehta and Mehta 2002, Boullion et al 1995, Campbell 1996). However, the use of 1,25(OH)₂D₃ in clinical practice is limited due to its severe toxicity at a concentration required to suppress cell growth. Therefore, numerous analogs of vitamin D have been synthesized and evaluated for efficacy and toxicity in a variety of models. Of these several hundred analogs, EB1089, RO24-5531, 22-oxa-calcitriol, 25-hydroxyvitamin D₃, and 1 α -hydroxyvitamin D₅ have been successfully used at relatively non-toxic concentrations in experimental in vivo carcinogenesis models and have progressed for evaluation in clinical trials. We synthesized 1 α (OH)D₅ a few years ago (Mehta et al 1997) as an analog of the vitamin D₅ series of compounds, since it was considered the least toxic in the series of vitamin D analogs (vitamin D₂ to vitamin D₇). As described later under the section, 'Preliminary Results,' we also showed that this analog could be tolerated at higher concentrations than any of the other efficacious analogs of vitamin D. It mediates its action via

vitamin D receptors, inhibits cell transformation, but does not affect normal breast epithelial cell growth.

Although the majority of the work with $1\alpha(\text{OH})\text{D}_5$ has been done with breast cancer cells and mammary carcinogenesis models (Mehta RR 2000), we evaluated its efficacy in LNCaP prostate cancer cells. Results showed antiproliferative effects of $1\alpha(\text{OH})\text{D}_5$ at 10^{-6}M concentration. In an *in vivo* study, LNCaP cells were inoculated in athymic mice and were treated either with vehicle or with 12.5 $\mu\text{g/kg}$ diet of $1\alpha(\text{OH})\text{D}_5$ for 8 weeks. Tumor size was measured weekly. Results showed that $1\alpha(\text{OH})\text{D}_5$ suppressed growth of LNCaP cells in athymic mice. These results indicate that $1\alpha(\text{OH})\text{D}_5$ may be efficacious against prostate cancer in addition to its activities against the breast cancer. The effect of $1\alpha(\text{OH})\text{D}_5$ in the experimental prostate carcinogenesis model has not been published. However, an experiment has recently been completed in our laboratories, where prostate cancer was induced in rats with MNU, and the animals were then treated with 50 $\mu\text{g/kg}$ $1\alpha(\text{OH})\text{D}_5$ -supplemented diet. Histopathological results from this study have not been evaluated (McCormick, Mehta, and Bosland: unpublished data), but soon will be available.

Prior to undergoing clinical evaluation, any compound has to be evaluated for safety and 'dose finding' in two species under Good Laboratory Practice regulations. We recently completed a preclinical toxicity study under a subcontract to IIT Research Institute (Dr. McCormick) to determine dose tolerance in Beagle dogs and Sprague Dawley rats. These preclinical toxicity results are described under a separate heading in this document [See Section 4].

Following is a list of preliminary results generated in our laboratories rationalizing the selection of the agents and procedures for the current application.

- We had reported synthesis of $1\alpha(\text{OH})\text{D}_5$ for initial studies, and since then it has been synthesized under good manufacturing practice (GMP) for Phase I clinical trials for breast cancer studies. This material is available and will be purchased from ConQuest Inc., Chicago.
- $1\alpha(\text{OH})\text{D}_5$ induces cell differentiation and inhibits cell proliferation of VDR+ breast cancer cells. *In vitro*, when breast cancer cells were exposed to $1\alpha(\text{OH})\text{D}_5$ (0.1-10 μM), an antiproliferative effect was observed. *In vitro* treatment for 7-10 days also showed induction of various biomarkers associated with breast cell differentiation (such as $\alpha 2$ integrin, ICAM-1, nm23 lipid accumulation, and accumulation of β casein) in breast cancer cells positive for VDR. VDR-/+ MDA-MB-231 only marginally responds. Breast cells (only VDR+) exposed to $1\alpha(\text{OH})\text{D}_5$ *in vitro* lost their tumorigenic ability when transplanted into mice.
- Prostate cancer cells sensitive to androgen, LNCaP cells, are VDR+ and respond to both $1\alpha,25$ -dihydroxyvitamin D₃ and $1\alpha(\text{OH})\text{D}_5$, with a similar growth responsiveness as MCF-7 cells.
- LNCaP cells also exhibit induction of VDR following incubation for 7 days with 1 μM $1\alpha(\text{OH})\text{D}_5$.

- Both $1\alpha(\text{OH})\text{D}_5$ and $1\alpha,25(\text{OH})_2\text{D}_3$ induced $\text{TGF}\beta_1$ in the alveolar cells of this tissue. 1α -Hydroxyvitamin D_5 was effective against MNU-induced rat mammary carcinogenesis. It inhibited both incidence and multiplicity in Sprague-Dawley rats at 25 and 50 $\mu\text{g/kg}$ diet without any hypercalcemic activity.
- $1\alpha(\text{OH})\text{D}_5$ at 12.5 $\mu\text{g/kg}$ diet inhibited growth of ZR75/A, T47-D, and BCA-4 cells in athymic mice. However, MDA-MB-231 cells did not respond to $1\alpha(\text{OH})\text{D}_5$.
- $1\alpha(\text{OH})\text{D}_5$ shows in vivo growth-inhibitory action on LNCaP prostate cancer cells. Preliminary studies show that $1\alpha(\text{OH})\text{D}_5$ inhibits *in vivo* growth of prostate cancer cells. An in vivo experiment was performed on a small group ($n=4$) of animals. Prostate cancer LNCaP cells were injected s.c. in 6- to 8-week-old male Balb/c athymic mice. Animals were given a control diet or a diet supplemented with 20 $\mu\text{g/kg}$ diet $1\alpha(\text{OH})\text{D}_5$. Eight weeks after treatment initiation, only 1/4 (25%) of $1\alpha(\text{OH})\text{D}_5$ -treated animals showed tumor development; in controls, 4/4 (100%) animals showed tumor development. Mean tumor volume in $1\alpha(\text{OH})\text{D}_5$ -treated animals ($n=2$ only developed tumor) was 0.06 cm^3 vs $0.15\pm0.05\text{ cm}^3$ ($n=4$) in control group. The PI realizes that our sample size is too small to determine statistical significance. However, the results shown here are preliminary in nature and suggest that $1\alpha(\text{OH})\text{D}_5$ could serve as a potential therapeutic agent for prostate cancer cells.
- Preclinical toxicity was determined in rats and dogs. The rats received 28 days gavage treatment of increasing concentrations of $1\alpha(\text{OH})\text{D}_5$ in a range of 2.5-10 $\mu\text{g/kg}$ body weight for CD-1 rats and 5-50 $\mu\text{g/kg}$ bodyweight for dogs. A complete battery of in-life, clinical pathology, and histopathology evaluations were performed. No toxicity or enhanced calcium levels were observed in rats. In beagle dogs, concentrations of 5 $\mu\text{g/kg}$ body weight resulted in no toxicity, whereas concentrations greater than 10 $\mu\text{g/kg}$ body weight resulted in loss of body weights, increased calcium, and gross toxicity. These results were utilized to develop a clinical Phase I trial protocol for breast cancer patients. We will be able to use these data for the proposed trial in this application. These maximum tolerated doses are considerable higher than $1\alpha,25$ -dihydroxy D_3 .
- $1\alpha(\text{OH})\text{D}_5$ has the potential to advance from the laboratory to the clinic. $1\alpha(\text{OH})\text{D}_5$ is scheduled to be used in a phase I clinical trial in breast cancer patients under a U.S. Army CTR breast cancer research award (# BC984013).

INTERMEDIATE BIOMARKERS IN PROSTATE CANCER

Selecting intermediate endpoint markers for the diagnosis, progression, or response to treatment for cancer patients has been a major challenge. In this respect, prostate cancer diagnosis has been considerably simplified by the examination of PIN and PSA. Numerous markers have been evaluated for a variety of chemopreventive agents for prostate cancer (Lazzaro, 2000). The intermediate biomarkers used for the two-cohorts in Phase II chemoprevention clinical trials include PIN (nuclear polymorphism, nucleolar size, and DNA ploidy), proliferation kinetics check points including PCNA, apoptosis, loss of heterozygosity,

and signal transduction markers including TGF α and β , IGF, c-erbB-2, and PSA levels. These markers have to be selected based on the progression of the disease as well as the chemopreventive agent. In a prostate cancer Phase II clinical trial with N-(4-hydroxyphenyl) retinamide, several additional markers were used, including p53, ploidy, and EGF receptors (Lazzaro, 2000).

RESEARCH METHODS

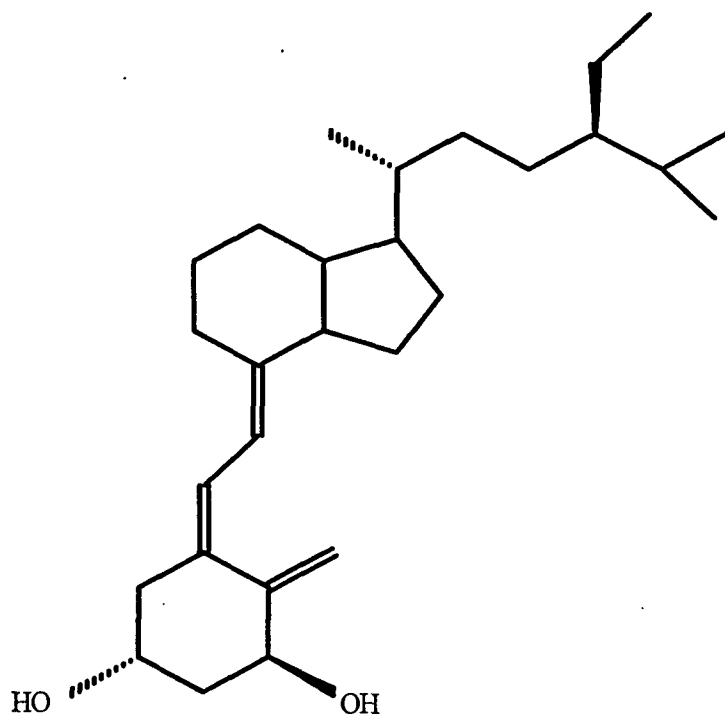
1 α -HYDROXYVITAMIN D5

The analog 1 α (OH)D₅ was synthesized by ConQuest, Inc. (Chicago, IL) under GMP (Good Manufacturing Practice) for the Phase I/II clinical trial for breast cancer patients and is available from Merrifield Pharma, Inc. (Westmont, IL) for the present study. We also have completed (as a subcontract to IIT Research Institute, Chicago) preclinical toxicity studies in two species. We will purchase 1 α (OH)D₅ from Merrifield Pharma, Inc. (ConQuest, Inc. was sold to United Therapeutics in 2000 and no longer manufactures D5.) Meeting the prerequisites for using a compound in a clinical setting is very crucial for the success of the project. The current study therefore can be implemented clinically without any delay, once FDA approval is obtained.

Physical, Chemical and Pharmaceutical Properties and Formulation

Chemical Information

1 α -Hydroxyvitamin D5 Structural Formula



Chemical Name: 1α -Hydroxyvitamin D₅ is a structural analog of vitamin D₅. The chemical name for it is 1α -Hydroxy-24-ethyl-cholecalciferol [$1\alpha(\text{OH})\text{D}_5$]

Synthesis: The compound has been synthesized by Dr. Raju Penmasta, Merrifield Pharma, Inc., Westmont, IL, according to the Good Manufacturing Practice guidelines.

Molecular formula: C₂₉H₄₈O₂

Molecular Weight: 428.6

Physical form: White powder

Solubility: It is insoluble in water but highly soluble in ethanol.

Purity: The acceptable limit for the purity of the substance is 95-100%, and the analytical method used to assure the identity and purity of the compound is reversed-phase HPLC. The compound, 1α -hydroxyvitamin D₅, was separated on a C18-reversed phase 75x 4.6 mm, 3.5 micron column using a mobile phase of 90% acetonitrile in water. 1α -Hydroxyvitamin D₅ was separated with a flow rate of 1 ml/min and monitored at 265 m μ . It was eluted with the retention time of 35 min.

PHARMACEUTICAL INFORMATION

The compound is being formulated in the form of an oral capsule. The concentration in each capsule will be created according to the protocol approved for the Phase I clinical trial. This will be comparable to the oral capsule given to animals in preclinical toxicity studies under Good Laboratory Practice guidelines. The capsules for each dose level will be prepared according to the dosage schedule at the time of the initiation of the study (Table 1). This will be prepared within the Pharmaceutical Science Department in the School of Pharmacy at the University of Illinois. All the inactive ingredients in the capsule will be standard pharmaceutical components, which comply with pharmacopeal guidelines. The capsules will be stored in a freezer to avoid degradation of vitamin D.

PRECLINICAL TOXICITY

The main reason new analogs of vitamin D are being developed is to generate compounds with reduced or no toxicity. The analog $1\alpha(\text{OH})\text{D}_5$ is one such relatively non-toxic vitamin D analog. We have completed an extensive series of preclinical toxicity studies for this vitamin D analog. In this section, we describe gross toxicity, calcemic activity in vitamin D deficient rats, and preclinical toxicity studies in two species, rats and dogs, under GLP.

GROSS TOXICITY

Treatment of animals with vitamin D analogs often results in loss of body weight. This is the first noticeable toxicity in animals. During the past few years, several experiments were performed where mice and rats were used as experimental models. As shown below, the tolerated doses for athymic mice, Balb/c mice, and Sprague Dawley rats were determined. These doses represent concentrations at which there was no loss of body weight or no adverse effects on general health. The animals were weighed twice per week and observed daily for lethargy and other noticeable changes.

MEASUREMENTS OF CALCEMIC ACTIVITY IN VITAMIN D-DEFICIENT RATS

Male rats three weeks of age were fed a diet containing 0.47g% calcium, 0.3g% phosphorus and no vitamin D. After three weeks of consumption of this diet, serum calcium levels were measured on selected animals. Animals exhibiting serum calcium values of less than 6.0 mg/dL were considered as vitamin D-deficient. The rats were treated with appropriate vitamin D analog for 14 days intragastrically. At the end of the study, the calcium concentrations were measured in the serum. The vehicle-treated control rats showed calcium concentrations of 5.4 ± 0.3 mg/dL (mean \pm standard deviation). When animals were injected with 0.042 μ g/kg/day of vitamin D analogs, the plasma calcium concentrations of 6.0 ± 0.6 mg/dL for $1\alpha(\text{OH})\text{D}_5$ (11% increase over control, statistically not significant from that of the control) and 8.1 ± 0.1 mg/dL for $1\alpha,25(\text{OH})_2\text{D}_3$ (50% increase over control, statistically significant) were observed. At a higher concentration of 0.25 μ g/kg/day, $1\alpha(\text{OH})\text{D}_5$ exhibited plasma calcium concentration of 8.1 ± 0.1 mg/dL as compared to 10.1 ± 1.8 for $1\alpha,25(\text{OH})_2\text{D}_3$. Although both analogs increased serum calcium in comparison to the control samples, these results showed overall lower calcemic effects for $1\alpha(\text{OH})\text{D}_5$ as compared to $1\alpha,25(\text{OH})_2\text{D}_3$.

Experiments were carried out to determine maximum tolerated dietary dose of $1\alpha(\text{OH})\text{D}_5$ for rats. Sprague-Dawley rats were separated into 11 groups of 10 animals each. Group 1 served as a control. Rats in other groups received either five doses (0.8, 1.6, 3.2, 6.4, and 12.8 g/kg) of $1,25(\text{OH})_2\text{D}_3$ or five doses (3.2, 6.4, 12.5, 25, and 50 g/kg) of $1(\text{OH})\text{D}_5$ for six weeks. Results showed that there was hypercalcemia and loss of body weight observed at 12.8 g/kg diet, whereas there was in fact increased body weight observed at 50 g/kg of $1\alpha(\text{OH})\text{D}_5$ dose level. In a separate study, there was no adverse effect of D_5 on the body weight gain observed at 100 g/kg diet. Therefore, the $1(\text{OH})\text{D}_5$ can be tolerated at a much higher concentration than the dihydroxy- D_3 analog of vitamin D.

Preclinical Toxicity (GLP)

Four-week oral (gavage) toxicity studies were performed on rats and dogs at the IIT Research Institute in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations as set forth in the *Code of Federal Regulations (21 CFR Part 58)*.

Studies in Rats

A 28-day toxicity study was performed in both male and female CD rats. Ten animals per sex per dose were entered in the study. 1α -Hydroxyvitamin D_5 was administered in corn oil at three dose levels: 2.5, 5.0, and 10 μ g/kg of body weight. A control group of rats received only vehicle. Ten additional animals were kept in control and high dose groups for a 14-day recovery period. All animals were observed for adverse clinical signs, body weight gain, and food consumption. Clinical pathology, hematology, and clinical chemistry measurements were carried out for every animal. All animals were subjected to gross necropsy, and tissues from control and high-dose animals were processed for histopathological evaluation. No animals died from the treatment during the study. No clinical signs or adverse toxicity-related symptoms were observed at any dose level. No effect on food consumption or body weight gain was observed during the study. Treatment-related increased calcium was observed in the high-dose group

(Control 11.0 ± 0.46 vs. high-dose 11.6 ± 0.73 mg/dL). Calcium and phosphorus were not increased in the recovery group of animals. Increased incidence of mineralization in the kidneys was observed at high doses. All microscopic changes were of minimal to mild severity. In summary, there was a minimal severity of mineralization observed in kidneys at high-dose level in both sexes. These lesions often occur as incidental findings in rodent studies. Therefore, although an absolute no-effect level dose was not established, minimal toxicity was observed in these experiments and that might not be 1α -hydroxyvitamin D5-related.

Four-Week Oral Toxicity Study in Beagle Dogs

A 28-day oral toxicity study was performed in both sexes of beagle dogs to evaluate the toxic effects of 1α -hydroxyvitamin D5. The vitamin D analog was administered in a vehicle of corn oil in 1 ml volume /kg/day at three dose levels of 10, 30, and 90 $\mu\text{g/kg/day}$. The vehicle was administered in the control group of dogs. Three dogs per sex per each concentration were entered in the study. Two additional dogs were kept for vehicle and high-dose group for a recovery experiment. However, because of mortality in high dose groups, the 2 dogs in the high dose recovery group were transferred to the toxicity study, and the 90 $\mu\text{g/kg}$ dose level was reduced to 45 $\mu\text{g/kg/day}$ for the remainder of the study. The two dogs from the recovery group of the control group were dosed 5 $\mu\text{g/kg/day}$ for 28 days. Toxicological endpoints included physical examination, clinical observations, ophthalmic examination, body weights, food consumption, hematology, clinical chemistry, electrocardiographic evaluations, and histopathological evaluations for all animals. Eight dogs died during the study: 2 females and 3 males at 90 $\mu\text{g/kg/day}$ dose, and 2 males and 1 female at 30 $\mu\text{g/kg/day}$. Toxicity was observed at all concentrations above 10 $\mu\text{g/kg/day}$. Serum calcium increased at concentrations of 10 $\mu\text{g/kg}$ and above. However, no ophthalmic or cardiac toxicity was observed at any dose level. In summary, the results indicated that dogs were more sensitive to 1α -hydroxyvitamin D5 as compared to rats, and the maximum tolerated dose for this analog in dogs was 5 $\mu\text{g/kg/day}$ or slightly higher but less than 10 $\mu\text{g/kg/day}$.

Summary:

Results described in this section have clearly defined the maximum tolerated dose levels of $1\alpha(\text{OH})\text{D}_5$ and compared it to the 'standard' active metabolite of vitamin D (1,25-dihydroxyvitamin D3). Results showed that the D5 analog could be tolerated at more than 10 times the concentration of 1,25 dihydroxy D3 without affecting body weight or hypercalcemic condition. The preclinical toxicity in two species is completed under GLP regulations and results have indicated that it is safe to evaluate $1\alpha(\text{OH})\text{D}_5$ for Phase I/II clinical trials.

PROCEDURES

FOLLOW-UP

1. Forty patients will be seen once every four months in the clinic, except in the initial 1-4 months as detailed in the box below. In 2003, the UC Davis Cancer Center saw 41 potentially eligible patients. In 2002, 28 potentially eligible patients were seen. Since

investigators will draw on four years during which patients are eligible for the study (12-60 months post-radiation therapy), there will be a large enough pool of patients from which to draw subjects for this study (about 140 patients who meet study inclusion and exclusion criteria). Generally, prostate cancer patients are seen every four months after they complete radiotherapy, so this part of the study schedule poses no additional burden on the patients.

2. Patients will have blood drawn for PSA prior to digital rectal examination (DRE).
3. Patients will have a complete history taken, and a physical examination and a DRE performed.
4. Patients' compliance will be documented [Pill Diary].
5. Pill Diary will be submitted by patients.
6. Patients' symptoms [if any] will be documented.
7. Quality of Life forms will be completed. The Health Survey SF-36V forms will be used, as they have been validated [see attachment] (Ware 1995; Ware 1994).
8. The American Urological Association (AUA) GU Symptom Scoring Scale form (Appendix VI), which has been validated (Barry 1992), will be used in every follow-up appointment and will indicate whether, upon completion of radiation therapy, cancer progression might be occurring. (This is a standard follow-up procedure for prostate cancer patients, not particular to this study.)

After informed consent is obtained, all 40 subjects will participate in a one-month run-in period, during which they will take one placebo pill per day. The investigators will look at the pill calendar that the patients have filled out, and count the number of pills left in the bottle at the end of the month to measure compliance; any subject who is not within 10% of the expected count will be considered non-compliant and will be withdrawn from the study. Since there are 28-31 days in a month, the 10% non-compliance threshold allows patients to miss at most three pills; if they miss four pills, they will not be able to participate in the study. (See Informed Consent form.)

NOTE: INTENSIVE FOLLOW-UP SCHEMA FOR THE FIRST PHASE TO IDENTIFY ANY UNUSUAL 'REACTORS'

- During the first month of the study, patients will be seen once a week and an interview for any toxicity will be done by the CRA and blood will be drawn for calcium levels.
- If any symptoms develop, they will be seen by a physician
- If calcium levels are elevated x 1.5 times the base line, a dose reduction to 50% of the dose will be done
- If calcium levels are stable in the first month, then patients will be seen once a month; a telephone call will be made once a week from month 2 to 4
- If calcium levels are stable during the first 4 months and if the patients are clinically stable without any toxicity, then they will be seen once in four months; once a month phone calls will be made during the second 4-month period.
- Phone call evaluations will be discontinued if patients are clinically and biochemically stable for the first 8 months.
- Weekly Evaluations of Calcium and Phosphorus in blood, PTH at baseline and once every four months.

REGULAR FOLLOW-UP VISITS

After the Initial Intensive Follow-up, patients will be seen once in four months, or earlier if necessary. These visits will be exactly the same as the first 4-month visit, which included a physical examination, a digital rectal examination, blood tests [about 13 cc's drawn from the patient's vein] and filling in some forms. These forms ask questions about the patient's quality of life; that is, whether there are any changes in his abilities or enjoyment. These are the same forms study subjects were asked to complete at the beginning of the study. Our Clinical Research Associate will help them to complete the forms if the patients have any questions.

Summary of Schedule for Study Participants (40 patients)									
Month(s):	Run-in		1		2-4		5, 9, 13, 17, 21		24
Event/Procedure	Week(s):		1	2-5	1	2-5	1	2-5	5
Informed Consent Given	X								
Clinic Visit	X	X	X	X	X			X	X
Physical exam by doctor	X							X	X
Digital Rectal Exam (DRE)	X							X	X
Complete History	X								
Lab Collection ¹	X	X						X	X
Clinical Research Associate (CRA) Interview	X	X	X	X	X			X	X
Quality of Life forms completed	X		X		X			X	X
AUA GU Symptom Scoring Scale completed	X				X			X	X
Karnofsky Performance Scale completed	X	X	X		X			X	X
Pill Calendar given	X		X		X			X	X
Placebo pills given	X								
Placebo pills taken daily	X	X							
Pill Calendar collected			X		X			X	X
Study pills given			X		X			X	X
Study pills taken daily			X	X	X	X		X	
Patient symptoms documented			X		X			X	X
PTH			X					X	
Biopsy (following initial biopsy at time of diagnosis)									X
Telephone Call by CRA				X	X	X			

¹ for PSA, calcium, phosphorus, albumin, Chem 7, and urine electrolytes

The end-of-study biopsies will be performed by UC Davis urologists. The tissue will be safely stored in the UC Davis Pathology Department.

WITHDRAWAL/TERMINATION FROM THE STUDY

A study subject may withdraw from the study at any time. Subjects should inform the Principal Investigator about their withdrawal from the study. Their participation is completely voluntary.

Their decision to no longer participate will not affect their current or future relations with their doctors or other health care providers in the university. The Clinical Research Associates will try to follow-up with those participants who decide to withdraw on the same schedule as those participating in the study. If the patients refuse to participate in this way, then the Clinical Research Associates will try to have phone contact with the former study participants.

Once a patient has withdrawn from a study, the Principal Investigator and other study members are no longer allowed to obtain any new information from the patient's medical records. They may continue to use patient information which was collected before the patient withdrew. Charts of patients who have withdrawn would be stored and maintained offsite (not kept with the active study charts), and the charts would be designated as "withdrawn".

There are a few circumstances under which the principal investigator would terminate a subject's participation in the study (other than serious side effects to the study medication). First, if the patient was non-compliant in taking the study medication, the Principal Investigator could terminate the subject's participation. Secondly, if the study subject is experiencing other serious medical conditions that would interfere with satisfactory continuation of the study, the Principal Investigator could terminate their further participation. As with the case when a subject chooses to withdraw, the Clinical Research Associates will try to follow-up with these participants on the same schedule as those participating in the study. If the patients refuse or are unable to participate in this way, then the Clinical Research Associates will try to have phone contact with them.

TREATMENT PLAN

Administration

The study medication will be dispensed monthly by the research nurse. All patients will receive a one-month supply of either D5 or the placebo at their monthly visit with the research nurse, along with the pill diary form to record their medication use. Both of the study arms will follow the same schedule of drug administration. As stated in the protocol, the standard dose of D5 will be 10 µg per capsule, taken once a day. It will be recommended to patients that they maintain a low calcium diet and avoid calcium-containing medications, such as Tums.

At each follow-up visit, an assessment of patient medication compliance will be made and recorded in the patient's medical record. Compliance will be recorded as the percentage of pills taken. To help in the assessment of compliance, it is required that patients keep a pill diary record (using the form provided to them) of their daily pill consumption. Prior to starting treatment, the patient will be provided with and instructed in the proper use of a pill diary (see Appendix XIII for this form). The patient will be instructed to return this diary at specified intervals during treatment and at each follow-up visit. This record will be checked for compliance by the investigator. The diary will be retained in the patient's record. The diary will act as source documentation. Patients who are non-compliant with diary use will be re-instructed in the use of the diary.

Discontinuation of Drug

Upon completion or discontinuation of D5 or placebo, the patient will be instructed to return all unused supply to the investigator for proper disposal.

Toxicity-Based Dose Modification Schedule for D5

Toxicity	Grade 3 or 4
1 st appearance	The patient will go on a drug holiday for one month or until the toxicity has been resolved to grade 0-1, whichever is longer, then continue at 50% of starting dose (i.e., 5 µg per day)
2 nd appearance	Interrupt for one month or until resolved to grade 0-1, whichever is longer, then continue at 50% of previous dose (i.e., 2.5 µg per day)
3 rd appearance	Interrupt for one month or until resolved to grade 0-1, whichever is longer, then continue at 50% of previous dose (i.e., 1.25 µg per day)
4 th appearance	Discontinue treatment permanently

Identifying Patients Who Develop Emotional Problems

If the regularly administered Quality of Life assessment on any study subject suggests any significant deterioration, including psychological status, the same de-escalation protocol will be followed as is done for patients who develop medical toxicity to D5: drug holiday for one month, followed by a 50% reduction in D5 dose, up to three times.

MONITORING OF STUDY

The study will be conducted according to Good Clinical Practice (GCP) guidelines. GCP is a standard for the design, conduct, performance, monitoring, auditing, recording, analysis, and reporting of clinical trials. The Good Clinical Practice Program is the focal point within FDA for Good Clinical Practice issues arising in human research trials regulated by FDA.

MEDICAL MONITOR REQUIREMENT

Per HSRRB requirements, a medical monitor is assigned to this study. The name and *curriculum vitae* of the medical monitor is provided. This individual is a qualified physician who is not associated with this particular protocol, is able to provide medical care to research subjects for conditions that may arise during the conduct of the study, and will monitor the subjects during the conduct of the study. The medical monitor is required to review all serious and unexpected adverse events (per ICH definitions) associated with the protocol and provide an unbiased written report of the event within 10 calendar days of the initial report. At a minimum, the medical monitor will comment on the outcomes of the adverse event (AE) and relationship of the AE to the test article. The medical monitor will also indicate whether he/she concurs with the details of the report provided by the study investigator.

The medical monitor for this study is Dr. Rachel Chou of U.C. Davis Cancer Center.

DATA SAFETY MONITORING BOARD

The UCD Data Safety and Monitoring Committee will review the data at least every six months and evaluate the results.

MONITORING OF SIDE EFFECTS DURING ONE-MONTH RUN-IN PERIOD

During the one-month run-in period, when study subjects are taking a placebo to judge their ability to comply with pill-taking requirements of the study, any side effects or adverse events will be monitored by the Clinical Research Associates. Since the study subjects will be taking a placebo, no side effects are anticipated. However, the study subjects will be given the phone numbers of all the relevant study personnel, including the Principal Investigator, other study physicians, and the Clinical Research Associates.

ACCOUNTABILITY PROCESS FOR THE STUDY DRUG

The UC Davis Cancer Center has an investigational drug service in its Pharmacy Department, headed by Victoria Bradley, Pharm.D. Investigational drugs, such as D5 for this study, are first sent directly to the Cancer Center Pharmacy, and then they manage the distribution of the drug. Study coordinators must fax a patient's consent form to the Pharmacy in order to receive the study drug. Files regularly are audited by the UC Davis Cancer Center Data Safety Monitoring Committee. The Pharmacy has a log system in place to keep track of all investigational drugs, which includes their receipt, storage, inventory, disposition, and the disposal of unused supplies.

ENDPOINTS OF THE STUDY

1. Proportion of Patients Having Rising PSA
(Three consecutive increases in PSA; ASTRO criteria, Shipley et al., 1999).
2. Proportion of Patients Having PSA Failure (and using other definitions, such as doubling time)

Definition of PSA failure is per Jani et al., *Urology*, 1999. Briefly, this definition derives from the observation that the logarithm of the PSA profile curve provides more applicable information about the natural history of failure than the PSA profile curve itself. This biochemical failure criterion is based on a quadratic curve fitting of the logarithm of the PSA profile. First, the logarithms of the follow-up PSAs are computed, and a quadratic curve, $fPSA$, is fitted through this log PSA profile. Biochemical failure is declared when the $fPSA$ is twice the fitted nadir. Since normal PSA values are not indicative of failure, if the fitted nadir PSA is 1 or less, biochemical failure is declared when $fPSA=2$.

3. Proportion of Patients with Cancer Present in End of Study Biopsy Specimens
4. Toxicity

5. Number of patients for whom drug discontinuation or dose reduction is required; median number of days on full dose of drug.

6. Quality of Life

See Appendix V for the Quality of Life form (6 pages). This will be administered every four months, during the regularly scheduled clinic visit.

7. Differences in Biomarkers Profile

Note: Patients on this study will continue to be followed beyond 2 years – as part of their regular cancer care, they will be followed until death or until study investigators lose contact with them. Only monitoring of toxicity and the end-of-study biopsy will cease after the 2 years of the study. Therefore, the clinical endpoint is indefinite.

STATISTICAL ANALYSIS

OVERVIEW

The primary aims of this study are to provide preliminary estimates of efficacy compared to placebo for design of a Phase III trial, and to assess the tolerability and safety of the vitamin D5 preparation. The study will be a randomized, double-blind intervention; randomization will use a permuted block design, stratified by baseline PSA level.

ANALYSIS OF PRIMARY ENDPOINTS

The proportion of patients having rising PSA (both ASTRO and Jani definitions) will be summarized separately for the patients receiving D5 and placebo, and compared using Fisher's exact test. The proportion with cancer present in End of Study biopsy specimens will be compared similarly. Efficacy analyses will be intent-to-treat, and one-sided hypothesis tests will be used at level 0.05.

The proportion of patients experiencing toxicity and a 95% confidence interval will be calculated separately for patients receiving D5 and placebo, and compared using Fisher's exact test. The proportion for whom drug discontinuation or dose reduction was required will be summarized and compared similarly. Median number of days taking the full dose of the drug will be compared using non-parametric tests (Wilcoxon rank sum if no censoring, log rank if censoring.)

ANALYSIS OF SECONDARY ENDPOINTS

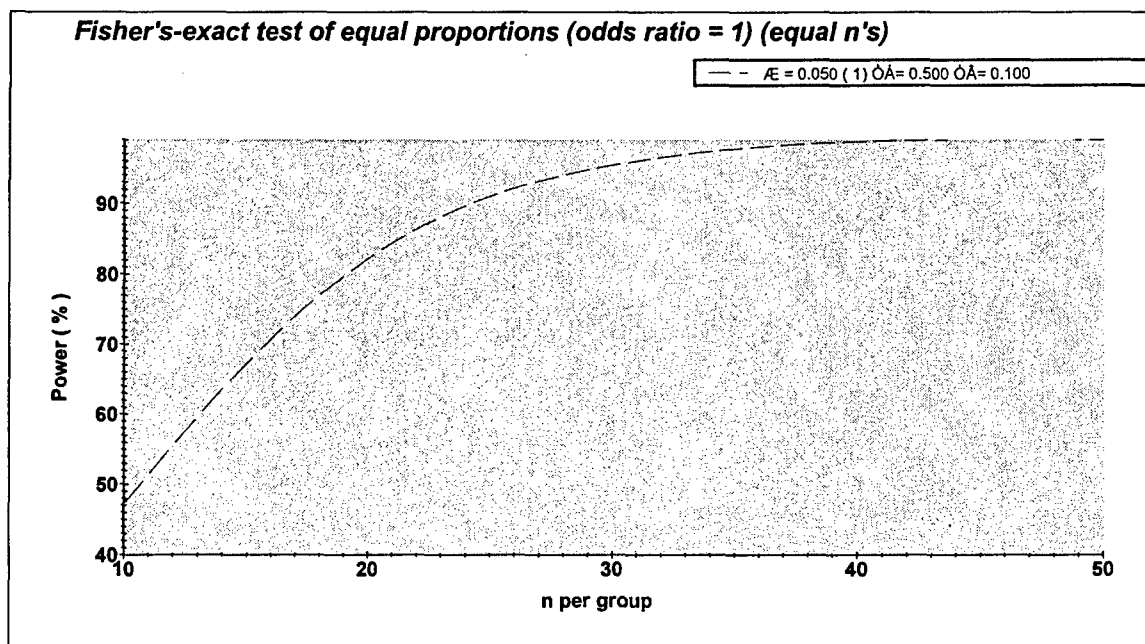
Both quality of life data and biomarker (PSA) data will be assessed every four months. Repeated measures regression models for longitudinal data (Laird and Ware, 1982) will be used to summarize the patterns of change in quality of life score and in biomarker measurement over the study period. The difference between overall mean level on treatment and the average rate of change per month will be estimated and compared for patients on Vitamin D5 vs. those on placebo. These models allow for the use of all available data, even if some measurements are

missing, and they allow for differences between individuals in baseline levels and rates of change, as well as within-person variation.

SAMPLE SIZE AND POWER CONSIDERATIONS

A sample size of 20 patients will be randomized to each group. The primary outcome will be recurrence of cancer, compared by one-sided Fisher's exact test at level 0.05. A one-sided test is appropriate because we will only consider a Phase III trial if there is evidence of efficacy. The proposed test will have 80% power to detect an improvement from a 50% recurrence rate with placebo (based on the literature) to a 10% rate with vitamin D5.

A sample size of 20 patients would ensure that we would observe, with 80% probability, at least one occurrence of any toxicity that occurred in at least 8% of patients, and with 90% probability any toxicity occurring in 11% or more of patients. We will be able to estimate the proportion requiring a dose reduction to at worst plus or minus 22% (based on 95% confidence interval and half of patients having difficulty tolerating dosage.) [Laird et al, 1982]



THE RATIONALE FOR THE $1\alpha(\text{OH})\text{D}_5$ DOSE IN OUR STUDY

TOXICITY OF CHEMOTHERAPEUTIC AGENTS

One pre-requisite in testing a chemotherapeutic agent in clinical studies is to conduct experiments in animal models to ascertain that the agent is effective at a non-toxic concentration (Mehta and Mehta, 2002). One primary side effect of vitamin D is hypercalcemia. Therefore, any analog of Vitamin D has to be shown to be active at non-hypercalcemic concentrations, or, even if it causes hypercalcemia, such an effect should be shown to be minimal. It is also important to mention that some analogs may be non-calcemic, yet may not be tolerated at high

concentrations, due to other toxicities. Therefore, in such cases, it is necessary to monitor the toxicity of the agent in a dose-response study.

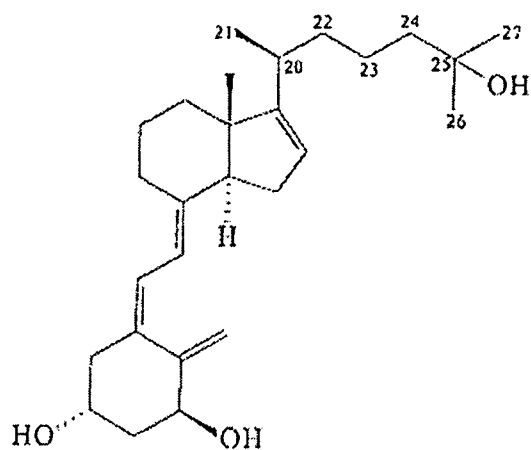
This is usually achieved by establishing a maximum tolerated dose [MTD] for each chemopreventive analog of the potential agent – in this case vitamin D. So far, some of the analogs of Vitamin D have been evaluated in vivo for their efficacy in chemoprevention. These include:

- RO24-5531 (Hoffman-LaRoche)
- EB 1089
- CB 966, MC903 (Leo Pharmaceuticals)
- 22-oxa-calcitriol (Chugai Pharmaceuticals Japan) and
- $1\alpha(\text{OH})\text{D}_5$ (OncQuest Inc.)

CHEMICAL STRUCTURES OF ANALOGS (FIGURE 1 ON THE NEXT PAGE)

FIGURE 1

R.G. Mehta, R.R. Mehta / Journal of Nutritional Biochemistry 13 (2002) 252-264



1α,25(OH)₂D₃

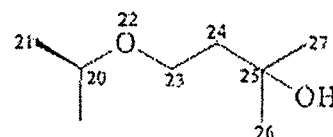
1. RO24-5531

R =



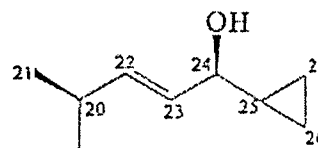
2. 22-Oxacalcitriol (OCT)

R =



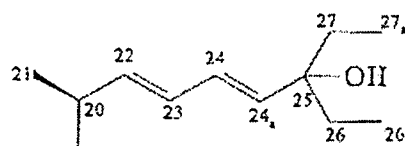
3. Calcipotriol (MC903)

R =



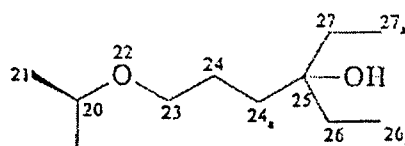
4. EB1089

R =



5. KH1060

R =



6. 1α-Hydroxyvitamin D₃

R =

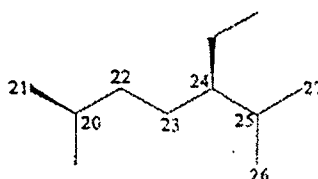


Fig. 3. Chemical structures of some of the active analogs of vitamin D.

EFFECTS OF VITAMIN D ANALOGS

The effects of vitamin D analogs have been studied mainly in mammary and colon carcinogenesis models to date. These results are summarized in Table 1.

Table 1: Summary of Efficacy of Vitamin D Analogs in Cancer Cell Proliferation (from Mehta RG and Mehta RR, 2002)

Table 1
Summary of efficacy of vitamin D analogs in cancer cell proliferation

Target organ	Cells	Vitamin D analogs	Efficacy	Comments
Breast	ER+			
	MCF-7, ZR75-1, T47D	22-oxa-calcitriol, $1\alpha(\text{OH})\text{D}_3$, EB-1089, KH11060, MC903, RO24-5531, 22-oxa-Calcitriol	All effective	VDR+
	BT474, BT20, SK-BR-3			
	ER-			
	MDA-MB-231, MDA-MB-436	$1\alpha(\text{OH})\text{D}_3$, 22-oxa-calcitriol, KH11060, RO24-5531	Ineffective	VDR+/
Prostate	UISO-BCA-4	$1\alpha(\text{OH})\text{D}_3$	Effective	VDR+
	UISO-BCA-1	$1\alpha(\text{OH})\text{D}_3$	Ineffective	VDR-
	MDA-MB-231	22-oxa-calcitriol	Effective	VDR+/
	Lncap, PC-3	$1\alpha(\text{OH})\text{D}_3$, EB1089, RO24-2637, 22-oxa-calcitriol, MC903	All Effective	VDR-
	Du-145	$1,25(\text{OH})_2\text{D}_3$, RO23-7553	Ineffective	VDR+/
Colon	Du-145	RO24-5531, RO26-2198	Effective	
	HT-29, CaCo-2	$1,25(\text{OH})_2\text{D}_3$, RO24-5531	Effective	VDR-

IN VIVO EFFECTS ON PROSTATE METASTATIC MODELS

There are at least two reports that establish the role of vitamin D analogs in preventing or retarding the metastasis of cancer cells to a distant organ as described below and thus clearly hint that these selective analogs may be very influential against the cancer cell metastasis:

1. Effects of 1,25-dihydroxyvitamin D₃ was evaluated and compared with EB1089 in transplantable prostate tumor model using androgen-insensitive metastatic rat prostate model. MAT LyLu cells were injected in Copenhagen rats and appropriate groups were treated with low (0.5 µg/kg) and high (1 µg/kg) doses. Both these analogs reduced the metastatic foci in lungs in these rats. However, this benefit was accompanied by hypercalcemia and loss of body weight at higher dose.
2. More recently, we evaluated effects of $1\alpha(\text{OH})\text{D}_3$ on the growth of LNCaP cells in athymic mice (unpublished) in our laboratories. Results showed that 55 nmole/kg (25 µg/kg) of the vitamin D analog D5 in the diet for 60 days resulted in reduced tumor volume as compared to the control LNCaP tumors. At 55 nmole/kg diet concentrations, the D5 analog did not elevate serum calcium levels. Thus, this experimental evidence indicates not only that these vitamin D analogs [D3 and D5] are effective as

chemopreventive agents in experimental [prostate] carcinogenesis models but also that they suppress the growth of human cancer cells in athymic mice [i.e., Cytostatic].

IN VIVO EFFECTS ON PROSTATE NON-METASTATIC MODELS

There are two studies conducted with $1\alpha(\text{OH})\text{D}_3$ in prostate non-metastatic models. One study is carried out in rats. In this model, prostate cancers are induced by MNU and treated with dietary modulation of 50 $\mu\text{g/kg}$ of $1\alpha(\text{OH})\text{D}_3$ for a two-year period. This study is just completed, awaiting histopathological evaluations (McCormick, Mehta, and Bosland in progress). A summary of these results is shown in Table 2.

Table 2: Effects of Vitamin D Analogs on Different Carcinogenic Models of Target Organs (from Mehta RG and Mehta RR, 2002)

Table 2

Summary of efficacy of vitamin D analogs in chemical carcinogenesis models

Organ	Models	Analog	Dose	Efficacy	Comments
Breast	MNU-induced adenocarcinoma	RO24-5531	1,10 nmole/kg diet	Effective	No toxicity
		1α -Hydroxyvitamin D_3	58.4, 116.8 nmole/kg	Effective	No hypercalcemia
				Dose related effect	No loss of body weight
		1α -hydroxy D_3	0.25 nmole	growth inhibition	Treatment schedule
		$1,25(\text{OH})_2\text{D}_3$	0.59-2.99 nmole/kg	No Effect	Hypercalcemia
		MC903	111 nmole/kg	Growth inhibition	Hypercalcemia
Prostate	MNU-induced	EB1089	1.1-5.5 nmole/kg	Effective	Hypercalcemia
					Loss of body weight
		RO24-5531	10 nmole/kg	Effective	No toxicity
Colon	AOM-induced DMH-induced DMH-induced DMH, MNU, and nitrosamines	$1\alpha(\text{OH})\text{D}_3$	58.4-116.8 nmol/kg diet	In progress	No effect on dorsal prostate
		RO24-5531	2.5 nmole/kg ip	Effective	No toxicity
		22-oxa-Calcitriol	72.5 nmole/kg ip	Effective	
		24R,25 dihydroxyvitamin D_3	0-24 nmole/kg	Effective	Reduced aberrant crypt foci colon only
		24R,25 dihydroxyvitamin D_3	0-12 nmole/kg	Effective	

Below, we have tabulated the MTD doses that have been established from animal studies and the type of toxicity for various Vitamin D Analogs:

Table 3: Maximum Tolerated Dose (MTD) Ranking for Commonly Used Vitamin D Analogs in Experimental Animals

Vitamin D Analog	Maximum Tolerated Dose (MTD)	Toxicity
1 α Hydroxyvitamin D5	116.8 nmole/kg diet	None
MC903	111 nmole/kg diet	Hypercalcemia
22-Oxacalcitriol	72.5 nmole/kg BW ip	None
24R,25 Dihydroxyvitamin D3	24 nmole/Kg BW	None
RO24-5531	10 nmole/kg diet	None
EB 1089	5.5 nmole/kg BW	Hypercalcemia
1,25 Dihydroxyvitamin D3	2.99 nmole/kg BW	Hypercalcemia
1 α Hydroxyvitamin D3	0.25 nmole/kg BW	None

It can be seen that D5's MTD is higher than all other analogs; also even at very high doses, no evidence of hypercalcemia has been demonstrated.

The above results show that:

1. 1 α (OH)D₅ is relatively well tolerated at much higher doses in experimental models than the doses of 10 μ g per day that is being planned in this clinical trial.
2. This dose is unlikely to pose any major clinical toxicity with long-term use, although the clinical trial is designed to carefully monitor the patients for any unexpected toxicity and to initiate either stoppage or dose reduction, if such unexpected toxicities occur.
3. As has been shown for 1,25(OH)₂D₃ [which has successfully reduced the rate of elevation of PSA in prostate cancer patients], it is reasonable to expect 1 α (OH)D₅ to be just as effective, but with fewer or no drug-related side effects/toxicities.

Finally, the Principal Investigator for this study, Dr. Srinivasan Vijayakumar, has previous experience with Phase II clinical trials (Vijayakumar 1993; Sweeney 1998).

2. SUBJECT SELECTION

ELIGIBILITY CRITERIA

1. Men who had received radiotherapy with curative intent. These patients should have had non-metastatic prostate cancer, i.e., no clinical or imaging evidence of distant metastases or lymph-nodal metastases. They should have been staged by standard procedures:
 - Digital Rectal Examination and documentation of the pre-RT findings in a AJCC Staging Sheet
 - Pre-treatment biopsy and a report of the grade of the lesion

- Pre treatment PSA levels (must be between 2 and 8 at the time of registration)
 - Bone Scan is recommended if the PSA level is over 15 ng/ml at the time of diagnosis [Chybowski et al., 1991; Vijayakumar et al., 1994]
2. The radiotherapy:
- Should have been completed within 5 years from the date of registration, but not within the immediate twelve months [see below]. Study entry criteria is based on clinical and biochemical status, so enrolling patients at different time periods after treatment will not cause a problem.
 - Could have been external beam RT [XRT] alone, XRT with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], brachytherapy alone, brachytherapy with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], or a combination of XRT and brachytherapy [again, if neoadjuvant hormonal therapy was given, it should have been for a duration not exceeding 12 months]
3. There should have been no evidence of metastatic disease at the time of diagnosis.
4. There should be no evidence of metastatic disease at the time of registration.
5. The PSA should have been stable [no more than 0.75 ng/ml variation in the PSA measurements], with at least 3 measurements within 12 months prior to the date of registration.
6. The Karnofsky Performance Status [KPS] should be 80% or more.
7. Patients have to sign an informed consent. They should be able to understand and consent in a fully informed document.
8. They should belong to Group II or III based on T-stage, Gleason Sum and PSA criteria:
- Group I = T1/T2 AND Gleason Sum <6 AND PSA < 10 ng/ml
 - Group II = One of the three factors higher than under Group I
 - Group III = Two or more of the three factors higher than under Group I
9. The age range of the subjects will be from 18 to 65+ years of age. There will be no maximum age limit for study subjects (Hall et al., 2004).

10. There are no medications and/or treatments, other than those listed in the inclusion/exclusion criteria, which study subjects must avoid due to the study medication.

INFORMED CONSENT PROCESS

Potential subjects will be patients from the clinics of the study investigators. The investigators will make the initial contact and will assess the inclusion/exclusion criteria for potential subjects using interviews. The discussion that the investigators will have with potential subjects will closely follow the text of the consent form (see attachment). The patient and his family will be given a consent form to take home and read, and will be encouraged to write down their questions. The patients will also receive a copy of this protocol. During the next patient visit, approximately one week later, the consent form will be discussed further, and the potential subjects will be asked to confirm that they have read the description of the study. They also will be able to discuss the study with their doctors until all questions are answered. Potential subjects will be asked to state that they understand: (1) that the study is to determine whether or not the treatment is effective and tolerated, as well as how effective it is; (2) that their participation is voluntary; and (3) that they know enough about the purpose, methods, risks, and benefits of the study to judge that they want to participate. Each potential subject must be able to provide informed consent, which will be obtained by the investigators.

ANONYMITY OF STUDY SUBJECTS

The anonymity of the study subjects will be maintained. In study records, subject names will not be used. Only initials will be used. No social security numbers will be used. Study coordinators will maintain a tracking book and be given a case study number for each study subject. *No identifiers, used for recruitment purposes, will be disclosed to a third party except as required by law or for authorized oversight of the research project.*

Any study records are going to be kept in a secure, locked cabinet in the Clinical Trials office. All of the University's data is password protected and only employees associated with the study will have access to them. Per University policy, study records will be maintained for 10 years.

EXCLUSION CRITERIA

1. Patients with metastatic disease.
2. Patients with a rising PSA as defined by the American Society for Therapeutic Radiology (ASTRO) criteria of three consecutive increases in PSA. PSA doubling time must be ≤ 6 months.
3. Patients who are on Androgen Deprivation Therapy.
4. Patients who are on 5-alpha reductase inhibitors such as Proscar. If they were on such therapy and discontinued at least 12 months prior to randomization, then they are eligible.
5. Patients with KPS less than 80%.
6. Patients with co-morbidities that lead to life expectancy of less than 5 years.
7. Patients who are unable to sign an informed consent.

8. Patients with other simultaneous or second malignancies within 5 years of registration.
9. Patients who had prostatectomies as part of treatment for prostate cancer or other conditions [for example, Abdomino-Perineal resection for rectal cancer].
10. PSA at registration exceeding a value of 10 ng/ml or less than 2 ng/ml.
11. Patients who are considering fathering children.
12. Patients who are unable to swallow and retain oral medicine.
13. Patients who would require a consent form that has to be translated into another language (i.e., a language other than English).
14. Patients with existing hypercalcemia.
15. Patients with existing hypercalciuria.
16. Patients with existing hyperparathyroidism.
17. Patients with existing sarcoidosis.
18. Patients with existing type distal renal tubular acidosis (type 1 RTA).
19. Patients with existing osteoporosis.
20. Patients with existing renal insufficiency (creatinine clearance $<60\text{mL/min}/1.72\text{m}^2$, based on the Cockcroft-Gault equation which allows the creatinine clearance to be estimated from the plasma creatinine in a patient with a stable plasma creatinine.

$$\text{CCr, in mL/min} = \frac{(140 - \text{age}) \times \text{lean body weight [kg]}}{\text{PCr [mg/dL]} \times 72}$$

21. Patients with a history of hypercalcemia while using vitamin D or vitamin D analogs.
22. Patients with a history of calcium containing kidney stones.
23. Patients with a history of hypercalemia related pancreatitis.

3. RISKS

INFORMED CONSENT PROCESS

Potential subjects will be patients from the clinics of the study investigators. The investigators will make the initial contact and will assess the inclusion/exclusion criteria for potential subjects using interviews. The discussion that the investigators will have with potential subjects will closely follow the text of the consent form (see attachment). The patient and his family will be given a consent form to take home and read, and will be encouraged to write down their questions. The patients will also receive a copy of this protocol. During the next patient visit approximately one week later, the consent form will be discussed further, and the potential subjects will be asked to confirm that they have read the description of the study or have had it translated into a language that they understand. They also will be able to discuss the study with their doctors until all questions are answered. Potential subjects will be asked to state that they understand: (1) that the study is to determine whether or not the treatment is effective and tolerated, rather than how effective it is; (2) that their participation is voluntary; and (3) that they know enough about the purpose, methods, risks, and benefits of the study to judge that they want to participate. Each potential subject must be able to provide informed consent, which will be obtained by the investigators.

Risks: Use of Specimens

There are very few risks to subjects. The greatest risk is the release of information from their health records, which may be necessary for investigators to obtain along with their specimens. Investigators will protect subjects' records so that their name, address, and phone number will be kept private.

Potential Risks and Discomforts

There are a number of potential risks and discomforts that subjects will be made aware of before they consent to participate. Subjects will be informed of any significant new findings developed during the course of the research that could affect their willingness to continue participation. The investigational agent to be used in this study is not approved by the Food and Drug Administration (FDA) for commercial use; however, FDA has permitted its use in this research study.

Potential Side Effects and Complications from the Study Medications

Although preliminary studies have indicated a relative safety of the Study Medication, one of the purposes of this study is to see whether there are any unexpected side effects from it. One of the known side effects of Vitamin D, when taken in excess or when the potent analogs are used, is an increase in blood calcium levels – this is called "Hypercalcemia". The symptoms of Hypercalcemia are listed in the tables below.

Some Potential Risks Associated with the Study Procedures		
<i>Procedures</i>	<i>Risks</i>	<i>Measures to Minimize Risks</i>
Taking the drug 1 α (OH)D5 for 2 years	Hypercalcemia: symptoms include loss of appetite, nausea, vomiting, abdominal pain, constipation, and other symptoms (see 2 nd table below). There may also be unknown effects since the study drug is a newly synthesized analog of vitamin D.	During the 2-year treatment period, subjects will be examined weekly, monthly, and then every 4 months. They also will be called by phone by the Clinical Research Associate weekly or monthly about the side effects they are experiencing, and the dosage of study drug will be adjusted or will be stopped temporarily or permanently as necessary.
Providing blood samples weekly, monthly, and every four months (2-3 tbsps each), over the course of two years	1) Pain, local bruising, bleeding, possible infection. 2) Possible breach of confidentiality.	1) Blood collection methods used in the study are the same as those used for routine clinical exams. 2) Procedures have been established for confidential collection, labeling, storage, use, and disposal of blood samples.
Ultrasound guided biopsy of prostate at the end of two years	Since needle biopsy will be used, risks are discomfort, local bleeding, small bruise, tenderness, infection (rare), and allergic reaction to local anesthesia.	
Completing "Quality of Life" survey several times during 2-year period	Inconvenience of completing forms	
Telephone interviews by Clinical Research Associate done weekly or monthly during 2-year period	Inconvenience of completing interviews	
Physical exam and digital rectal exam by radiation oncologist several times during 2-year period	Minor discomfort	

Symptoms of Hypercalcemia can be:

- Loss of appetite, nausea, vomiting, abdominal pain, constipation, inflammation of pancreas, stomach or intestinal ulcers
- Confusion, memory loss, tiredness, depression, even fainting
- Excessive urination, more frequent urination, including at night, kidney stone formation
- Muscle weakness, muscle aches, bone pain
- Increase in blood pressure, calcium deposits in the soft tissues of the body, a band formation in the cornea of the eye
- Itching
- HOWEVER, MOST PATIENTS DO NOT HAVE ANY SYMPTOMS. THAT IS ONE OF THE REASONS WE HAVE DESIGNED THIS STUDY WITH A PERIOD OF INTENSIVE FOLLOW-UP IN THE INITIAL FOUR MONTHS: TO IDENTIFY ANY OF THESE SYMPTOMS EARLY AND INTERVENE IF NECESSARY.
- ALSO, DEVELOPING SYMPTOMS DEPENDS UPON HOW LONG AND HOW RAPIDLY CALCIUM LEVELS INCREASE IN THE BLOOD. THE SHORTER THE DURATION AND LESS RAPID THE INCREASE, THE LESS ARE THE CHANCES OF DEVELOPING SIDE EFFECTS. THAT IS WHY, AGAIN, WE HAVE DESIGNED THE INTENSIVE FOLLOW-UP PERIOD TO DETECT ANY HYPERCALCEMIA AS SOON AS POSSIBLE, IF IT OCCURS.
- There may be other unknown and unexpected complications that could occur, including life-threatening complications.

Blood Drawing

The most frequent risks are bruising, pain at the site of needle stick, bleeding, and infection. The amount of blood drawn is unlikely to lead to anemia (low blood cell count).

Follow-up visits and completion of forms

Generally, prostate cancer patients are seen every four months after they complete radiotherapy, undergo a doctor's examination (including a digital rectal examination), and get blood drawn at the time of follow-up visits for PSA. So the follow-up schedule for the study is not any different than in other patients except during the initial phases. In addition, the number of telephone calls and the necessity of completing many forms can be inconvenient and may interfere with subjects' routine life.

Biopsy

This has the same risks and discomforts as the biopsy subjects had at the time of their diagnosis: A needle biopsy can be painful. Risks include bleeding and infection. Subjects may notice blood in their urine, in their semen, or with a bowel movement for several weeks after the biopsy.

What if a subject is injured as a result of participation?

All forms of medical diagnosis, treatment, and research, whether routine or experimental, involve some risk of injury. In spite of all precautions, subjects might develop complications from participation in this study.

If subjects are hurt or get sick because of this research study, they can receive medical care at an Army hospital or clinic free of charge. They will only be treated for injuries that are directly caused by the research study. The Army will not pay for subjects' transportation to and from the hospital or clinic. If subjects have questions about this medical care, they should talk to the principal investigator for this study, Dr. Srinivasan Vijayakumar, at (916) 734-7888. If subjects pay out-of-pocket for medical care elsewhere for injuries caused by this research study, contact the principal investigator. If the issue cannot be resolved, contact the U.S. Army Medical Research and Materiel Command (USAMRMC) Office of the Staff Judge Advocate (legal office) at (301) 619-7663/2221.

Subjects may, if they wish, receive treatment for a research-related injury at the UCD Medical Center. There is no compensation and/or payment for such medical treatment from the UCD Medical Center for such injury except as may be required of the University by law.

Should subjects feel they have been injured, they may contact:

- Dr. Vijayakumar, Principal Investigator, at (916) 734-7888
- Dr. Narayan at (916) 734-8051
- Dr. Ryu at (916) 734-8251
- Any of our Clinical Research Associates:
 - Clinical Research Nurse (to be named)
 - Cheri Koppe at (916) 734-3604
 - Cathy Hollister at (916) 734-8814

All routine diagnostic laboratory tests and follow-up office visit costs necessary for subjects' treatment will be borne by their insurance company (i.e., HMO or other health benefit provider). However, if their insurance company refuses to reimburse them, then subjects will be billed for these procedures. There will be no charge for the drug(s) or some of the specific tests performed to gather scientific information regarding this form of vitamin D. The biopsy at the end of the study carries the same risks as the biopsy subjects had at the time of diagnosis, and will not them you any additional expense.

REGULATORY AND REPORTING REQUIREMENTS

Adverse events (AE) reporting for this study is via AdEERS (Adverse Event Expedited Reporting System) and will follow the procedures presented in the "NCI Guidelines: Expedited Adverse Event Reporting Requirements for NCI Investigational Agents", which can be downloaded from the CTEP website (<http://ctep.cancer.gov/reporting/ctc.html>).

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0, will be utilized for adverse event reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 3.0. A table showing the **expected** adverse events specific for D5 is found below.

POTENTIAL SIDE EFFECTS AND COMPLICATIONS FROM THE STUDY MEDICATIONS

Although animal studies have indicated a relative safety of the Study Medication D5, and determined its relative safety compared to other D analogs (see Table 3), one of the purposes of this study is to see whether there are any unexpected side effects from it. One of the known side effects of Vitamin D, when taken in excess or when the potent analogs are used, is an increase in blood calcium levels – this is called "Hypercalcemia". The symptoms of Hypercalcemia are listed in the table below:

Symptoms of Hypercalcemia can be:

- Loss of appetite, nausea, vomiting, abdominal pain, constipation, inflammation of pancreas, stomach or intestinal ulcers
- Confusion, memory loss, tiredness, depression, even fainting
- Excessive urination, more frequent urination, including at night, kidney stone formation
- Muscle weakness, muscle aches, bone pain
- Increase in blood pressure, calcium deposits in the soft tissues of the body, a band formation in the cornea of the eye
- Itching
- However, most patients do not have any symptoms. That is one of the reasons we have designed this study with a period of intensive follow-up in the initial four months: to identify any of these symptoms early and intervene if necessary.
- Also, the development of symptoms depends upon how long and how rapidly calcium levels increase in the blood. The shorter the duration and less rapid the increase, the less are the chances of development of side effects. That is why, again, we have designed the intensive follow-up period to detect any hypercalcemia as soon as possible, if it occurs.
- There may be other unknown and unexpected complications that could occur, including life-threatening complications.

Expedited reports are submitted to CTEP via the secure AdEERS application accessed via the CTEP website ([https://webapps.ctep.nci.nih.gov/openapps/plsql/gadeers_main\\$.startup](https://webapps.ctep.nci.nih.gov/openapps/plsql/gadeers_main$.startup)) or, if it is impossible to access AdEERS, the paper templates that are available at <http://ctep.cancer.gov/forms/index.html> may be used.

REGULATORY REPORTING FOR INVESTIGATIONAL AGENTS

This study will utilize CTCAE version 3.0 for toxicity.

Submission of AEs

Adverse Events (AEs) for investigational agents should be submitted to CTEP via the web-based Adverse Events Expedited Reporting System (AdeERS).

In addition, adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (301-619-2165) (non-duty hours call 301-619-2165 and send information by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. Address the written report to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

Reporting Requirements for AEs

Reporting requirements and time of reporting are dependent on the phase of trial, grade and attribution and whether the event is expected or unexpected as determined by the NCI Agent Specific Expected Adverse Event List, protocol and or available Investigator's brochure.

UCD Responsibilities Regarding AEs

It is the responsibility of UCD to report all adverse events to the Investigational Drug Branch (IDB), Division of Cancer Therapy (DCT) via AdeERS or the expedited adverse event report Single Agent or Multiple Agents paper templates (available on the CTEP Home Page, <http://ctep.cancer.gov>). Reports are to be submitted within the timeframes specified.

In addition, adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy for Regulatory Compliance and Quality (see above).

AEs to be Reported to NCI Using AdeERS, and to USAMRMC

- all grade 4 and 5 AEs, including unrelated or unlikely unexpected and expected events
- any unexpected grade 2-5 AE (an expedited report is required for unexpected grade 2 and 3 AEs with an attribution of possible, probable or definite)
- any death within 30 days of drug administration
- any hospitalization (or prolongation of existing hospitalization) for medical events equivalent to CTC grade 3, 4, 5

Attribution Categories

Definite -- the adverse event is clearly related to the investigational agent(s)

Probable -- the adverse event is likely related to the investigational agent(s)

Possible -- the adverse event may be related to the investigational agent(s)

Unlikely -- the adverse event is doubtfully related to the investigational agent(s)

Unrelated -- the adverse event is clearly NOT related to the investigational agents(s)

Where to Send Copies of the Report

A copy of the submitted report must be sent to the Principal Investigator, Dr. Srinivasan Vijayakumar, by fax (916) 734-7076 or by e-mail (vijay@ucdavis.edu) for distribution to all participating study physicians, nurses and coordinators. The Adverse Event Report Form (Appendix IV) should be sent to the Principal Investigator within 24 hours. Any supporting documentation (i.e., laboratory, pathology, progress notes, discharge summary, autopsy, etc.) explaining the AE should accompany the submitted report.

Handling Questions About AEs

If there is any question about whether a particular adverse reaction should be reported, you may assess the event using AdEERS. The DCT encourages submission of reports even if there is only a suspicion of a drug effect.

Questions regarding AE reporting should be directed to the Clinical Research Associates, Cheri Koppe (916-734-3604, beeper 916-762-1601) or Cathy Hollister (916-734-8814, beeper 916-762-6282).

MEDICAL MONITOR REQUIREMENT

Per HSRRB requirements, a medical monitor is assigned to this study. The name and *curriculum vitae* of the medical monitor is provided. This individual is a qualified physician who is not associated with this particular protocol, is able to provide medical care to research subjects for conditions that may arise during the conduct of the study, and will monitor the subjects during the conduct of the study. The medical monitor is required to review all serious and unexpected adverse events (per ICH definitions) associated with the protocol and provide an unbiased written report of the event within 10 calendar days of the initial report. At a minimum, the medical monitor will comment on the outcomes of the adverse event (AE) and relationship of the AE to the test article. The medical monitor will also indicate whether he/she concurs with the details of the report provided by the study investigator.

The medical monitor for this study is Dr. Rachel Chou of U.C. Davis Cancer Center.

MONITORING OF SIDE EFFECTS DURING ONE-MONTH RUN-IN PERIOD

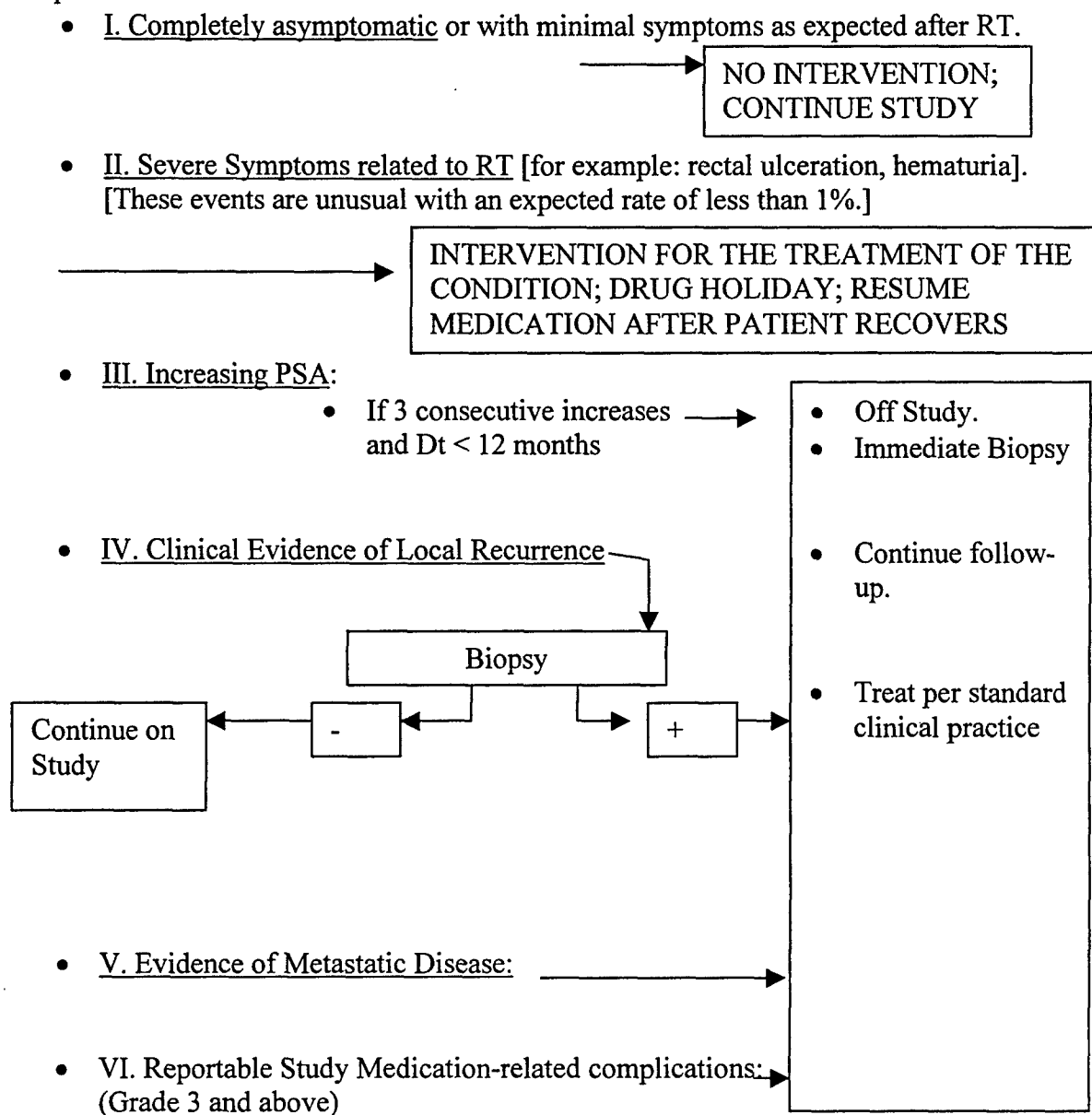
During the one-month run-in period, when study subjects are taking a placebo to judge their ability to comply with pill-taking requirements of the study, any side effects or adverse events will be monitored by the Clinical Research Associates. Since the study subjects will be taking a placebo, no side effects are anticipated. However, the study subjects will be given the phone numbers of all the relevant study personnel, including the Principal Investigator, other study physicians, and the Clinical Research Associates.

MODIFICATION OF PROTOCOL

The Principal investigator does not expect that the protocol will be modified and terminated, or extended. However, should there be a need for one of these to occur, the Principal Investigator will make such changes only with UCD IRB approval, the consent of the Cancer Center Data Monitoring Committee, and the Department of Defense. Any protocol modification is to be reviewed and approved by the HSRRB of the DOD prior to implementation of the modification. Similarly, HSRRB will be notified of any deviations from the protocol.

INTERVENTIONS DURING FOLLOW-UP PERIOD

The expected course of events is:



There is no rescue medication for this study. Study subjects experiencing adverse effects from the study medication (D5) will stop taking D5 and be provided necessary clinical support.

Most research-related injuries will be treated and resolved by the research institution, UC Davis Medical Center, which will follow its own policy for emergency care, as related in the informed consent form to the subject. In the event of a subject's needing non-emergency care, the PI will call the Army if the PI has a subject with a research-related injury that the PI's institution is unwilling to treat, or if the subject for some reason wants to explore Army treatment (at an Army Medical Treatment Facility) even though the institution has offered treatment.

The PI will be able to tell the subject where the nearest Army MTF is by looking at this website for a list: <http://www.armymedicine.army.mil/default2.htm> (click on Leaders and Organizations, then under Organizations, click on US Army Medical Department Organization Chart, then click on See All Online Army Medical Facilities). The PI cannot promise medical care from that Army MTF as the PI is not the one who will be making determination of eligibility. The PI will inform the study subject that if the Army finds him eligible for Army MTF care (because the Army agrees that the injury is research-related), then it is possible that subject can get medical care at an Army MTF. However, the subject should not call the Army MTF directly, because that is not how eligibility will be determined.

4. BENEFITS

Subjects may receive no direct benefit for participation in this study. Their participation will help other patients if Vitamin D5 is found to be an effective drug in preventing prostate cancer recurrence. As subjects will be randomized to treatment and control groups, ~50% of participants will receive D5. Those subjects would directly benefit from the hypothetical reduction in prostate cancer recurrence resulting from D5. Thus this research with Vitamin D5 might help people who have prostate cancer and other cancers in the future. The benefits of this research include improved understanding of prostate cancer treatment, recurrence prevention, and prophylaxis.

5. RISK-BENEFIT RATIO

This study poses minimal risk to participants and large potential benefit to future prostate cancer patients. Vitamin D5 has been shown to be safe and tolerable in animal models using doses in excess of several times the proposed dose used in this study. Furthermore, participants will be strictly monitored and followed for the development of any side effects or adverse reactions due to the administration of D5. It is our opinion that Vitamin D5 is a safe medication and is highly unlikely to result in significant side effects or adverse reactions. Vitamin D5 has also demonstrated anti-tumor activity against prostate cancer cell lines using both in vitro as well as in vivo animal models. It is our hypothesis that this effect will translate into reduction in the recurrence of prostate cancer in individuals who are at high risk to recur. No treatment modalities for the prevention of prostate cancer recurrence are currently available. Vitamin D5 may represent significant preventive treatment and ultimately provide direct benefit, measurable in reduced recurrence rates, in the ~50% of participants randomized to receive D5 treatment. As the theoretical risks to the administration of vitamin D5 are low and adequate steps have been

undertaken to recognize and manage these risks, it is our opinion that the treatment arm is at low risk in this study. The placebo arm, by nature of the study design is at even lower risk of side effects or complications. It is also our hypothesis that vitamin D5 will provide direct benefits to those patients randomized to the treatment arm. If D5 is effective in preventing prostate cancer recurrence the large potential benefit to future prostate cancer patients would be immeasurable. It is therefore our opinion that the benefits of undertaking this study of vitamin D5 far outweigh the risks.

6. COSTS TO SUBJECTS

Subjects will not be charged or paid to participate in the study. The study medications will be provided to subjects free of cost. The routine blood tests that are part of their regular follow-up will be paid by either the insurance company or by the patient, as in the case of a patient who had received radiotherapy and was being followed by his doctors. Subjects will not be charged for any of the particular blood tests that are specifically designed for the study.

It is possible that their insurance will not pay for all of the treatments and tests subjects will receive if they participate in the research. That is because many insurance companies, HMOs, and health benefits plans do not cover experimental treatments. Subjects will give us permission to submit bills to any appropriate third parties (insurance carriers).

All routine diagnostic laboratory tests and follow-up office visit costs necessary for subjects' treatment will be borne by their insurance company (i.e., HMO or other health benefit provider). However, if their insurance company refuses to reimburse subjects, then they will be billed for these procedures. There will be no charge for the drug(s) or some of the specific tests performed to gather scientific information regarding this form of vitamin D. The biopsy at the end of the study carries the same risks as the biopsy subjects had at the time of diagnosis, and will not be charged to the patient.

As stated above, if subjects are hurt or get sick because of this research study, they can receive medical care at an Army hospital or clinic free of charge. Subjects will only be treated for injuries that are directly caused by the research study. The Army will not pay for participants' transportation to and from the hospital or clinic.

7. DISCLOSURE OF PERSONAL AND FINANCIAL INTEREST IN THE RESEARCH STUDY AND SPONSOR

The principal investigator, co-investigators and sponsoring agency, the Department of Defense, have no personal or financial interests in this research study.

8. RESOURCES

The Department of Defense (DOD) has given the principal investigator a grant to conduct this study. The detailed budget given to the DOD shows that adequate funds have been allotted for personnel (% of time for principal investigator, co-investigators, research nurse, statistician), consultants, travel, subject-related costs, and other expenses.

Srinivasan Vijayakumar, M.D. Dr. Vijayakumar serves as the PI for this project. He is responsible for the overall project. Specifically, he is responsible for the clinical protocol, which will include all aspects of the radiation therapy and treatment with vitamin D5, follow-up, and pathology as well as clinical chemistry. Dr. Vijayakumar will spend 5% of his time on the project.

Ralph deVere White, M.D. Dr. deVere White will serve as Urologist on the project, assisting Dr. Vijayakumar with the clinical studies and obtaining biopsies. Dr. de Vere White will spend 2% of his time on the project.

Research Nurse (TBN). A nurse will assist Dr. Vijayakumar with the clinical studies. S/he will spend 25% of her/his time on the project.

Laurel Beckett, Ph.D., Statistician. Dr. Beckett will assist with the experimental design, sample size, and statistical analyses. She will be used on an as-needed basis with an effort commitment of 1% to 1.5% per year.

The following co-investigators will spend less than 1% of their time on the project:

Ralph Green, M.D., Pathologist. Dr. Green will collaborate on the project for the purposes of identification of PIN and other pathological conditions.

Samir Narayan, M.D.; Janice Ryu, M.D.; William Baker, M.D. These co-investigators will enroll patients into the clinical trial.

Paul Gumerlock, M.D. Dr. Gumerlock will assist with this study as it relates to the genetics of prostate cancer.

Rajendra Mehta, Ph.D. and Dr. Rajeshwari Mehta, Ph.D. These two co-investigators will conduct preliminary studies with D5, share their expertise in developing the appropriate doses of D5 for humans, and analyze data from the project.

Alan Diamond, Ph.D. Dr. Diamond will provide nutritional advice to the project, as needed.

Cathy Hollister and Cheri Koppe, Clinical Research Associates. Ms. Hollister and Ms. Koppe will assist with coordination of the project, as needed (the Clinical Research Nurse will have primary responsibility for this).

In addition, investigators have the invaluable resource of the U.C. Davis Cancer Center, where the study is being conducted. There is no cost to study participants. There is no compensation for participating in the study.

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ABBREVIATIONS

1 α (OH)D₅ = 1 α -Hydroxyvitamin D₅, 1 α hydroxy-24-ethyl-cholecalciferol, A vitamin D analog synthesized at the University of Illinois at Chicago

μ g = Micrograms

AdEERS = Adverse Event Expedited Reporting System

AE = Adverse Event

AI = Adequate Intake

ASTRO = American Society for Therapeutic Radiology

CRA = Clinical Research Associate

CTC = Common Toxicity Criteria

CTCAE = Common Terminology Criteria for Adverse Events

DCT = Division of Cancer Therapy

DOD = Department of Defense

DRE = Digital Rectal Examination

DU-145 = Prostate cancer cell line

FDA = Food and Drug Administration

GCP = Good Clinical Practice

GLP = Good Laboratory Practice

GMP = Good Manufacturing Practice

HSRRB = Human Subjects Research Review Board (of DOD)

ICH = the International Conference on Harmonisation of Technical Requirements for
Registration of Pharmaceuticals for Human Use.

IDB = Investigational Drug Branch

IU = International Units

LNCaP = Prostate cancer cell line

Mcg = micrograms

MNU = Methyl nitrosourea

MTD = Maximum Tolerated Dose

NIH = National Institutes of Health

PC-3 = Prostate cancer cell line

PSMA = Prostate-Specific Membrane Antigen

PSA = Prostate-Specific Antigen

QOL = Quality of Life

RDA = Recommended Dietary Allowance

RT = Radiation Therapy

TBN = To Be Named

TGF = Transforming growth factor

UCD = University of California, Davis

UCDMC = University of California, Davis Medical Center

UL = Upper Intake Level

USAMRMC = U.S. Army Medical Research and Materiel Command

UV = ultraviolet

VDR = Vitamin D receptor

VDRE = Vitamin D response element

APPENDIX I**KARNOFSKY PERFORMANCE SCALE**

Patient I.D. Sticker:

SCORE	DESCRIPTION
100	Normal, no complaints, no evidence of disease
90	Able to carry on normal activity; minor signs or symptoms of disease
80	Normal activity with effort; some signs or symptoms of disease
70	Cares for self, unable to carry on normal activity or do active work
60	Requires occasional assistance, but is able to care for most of his/her needs
50	Requires considerable assistance and frequent medical care
40	Disabled, requires special care and assistance
30	Severely disabled, hospitalization indicated Death not imminent
20	Very sick, hospitalization indicated. Death not imminent
10	Moribund, fatal processes progressing rapidly
0	Death

APPENDIX II**STAGING CRITERIA**

Patient I.D. Sticker:

DEFINITIONS**Tumor (T), Node (N), Metastases (M)
Classification Prostate Cancer****Primary Tumor (T)**

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor not palpable or visible by imaging
T1a	Tumor incidental histologic finding in 5 % or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5 % of tissue resected
T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA)
T2	Palpable tumor confined within prostate*
T2a	Tumor involves half of a lobe
T2b	Tumor involves more than half of a lobe, but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostatic capsule **
T3a	Unilateral extracapsular extension
T3b	Bilateral extracapsular extension
T3c	Tumor invades seminal vesicle
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles
T4a	Tumor external sphincter and/or bladder neck and /or rectum
T4b	Tumor invades levator muscles and/or is fixed to pelvic wall

Lymph Node (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional node metastasis
N1	Metastasis in a single lymph node, 2 cm or less in greatest dimension
N2	Metastasis in a single lymph node, more than 2 cm but not more than 5 cm greatest dimension or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension

Distant Metastasis (M) ***

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

M1a Non-regional lymph nodes
M2b Bone
M3c Other sites

- * Note: Tumor found in one or both lobes by needle biopsy, but not palpable or visible by imaging is classified as T1c
- ** **Note: Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3, but as T2**
- *** **Note: When more than one site of metastasis is present, the most advanced category (M1c) is used.**

APPENDIX III

COMMON TOXICITY CRITERIA (CTC)

Side effects will be based on NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0, which can be found at the CTEP website: <http://ctep.cancer.gov/reporting/ctc.html>.

APPENDIX IV
ADVERSE EVENT REPORT FORM

A Phase I/II Double-Blinded, Randomized Clinical Trial to Prevent/Delay Biochemical and Clinical Failure in High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1 α -Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding and Intermediate Biomarker Response-Seeking Study (HSRRB Log Number: A-11241)

Most frequently expected adverse events for this study:

Hypercalcemia:

- Loss of appetite, nausea, vomiting, abdominal pain, constipation, inflammation of pancreas, stomach or intestinal ulcers
- Confusion, memory loss, tiredness, depression, even fainting
- Excessive urination, more frequent urination, including at night, kidney stone formation
- Muscle weakness, muscle aches, bone pain
- Increase in blood pressure, calcium deposits in the soft tissues of the body, a band formation in the cornea of the eye
- Itching

1) Participant I.D. No: _____ 2) Protocol No: _____

3) Participant Initials: _____ 4) Investigator: Srinivasan Vijayakumar, MD

5) Institution Name: U.C. Davis Medical Center

6) Person Completing Form: _____
(Name & Signature)

7) Telephone: _____ Role in Study: _____

8) Randomization Date: _____

9) Study Drug ID number: _____

10) Still taking study drug?: ☐ Yes ☐ No, Date Discontinued: _____

11) Toxicity (per CTC): _____ 12) Toxicity grade: _____

13) Toxicity Category (choose one): ☐ Known ☐ Unknown ☐ Death

14) Attribution: Event related to study drug?
☐ Definitely ☐ Probably ☐ Possibly ☐ Not Likely ☐ Definitely Not

15) Date of Adverse Event Started: _____

ADVERSE EVENT REPORT FORM (page 2)

16) Date of Adverse Event Ended: _____

17) Toxicity Description: _____

_____18) Pre-existing Conditions: (describe all that apply): _____

19) Number of subjects enrolled to date: _____

20) Number and type of serious and unexpected adverse events reported previously in the study: _____

_____21) Description of the Study (e.g., double or single blind; phase of study the subject is participating in): _____

_____22) Synopsis of the Event: _____

_____23) Status of the subject: _____

_____24) Actions taken in response to this event: _____

_____25) Resolution of the adverse event (include modifications/changes to protocol): _____

Signature of Investigator_____
Date

APPENDIX V**QUALITY OF LIFE FORM****Sticker:****HEALTH SURVEY SF-36V**

Instructions: Please read each question and fill in the box that best describes your experience.

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.

Answer every question by marking with an "X" the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

Date of form completed: _____

1. In general, would you say your health is:

- ☐ Excellent
- ☐ Very Good
- ☐ Good
- ☐ Fair
- ☐ Poor

(continued on next page)

2. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

(SELECT ONE ANSWER FOR EACH QUESTION)

ACTIVITIES	Yes, limited a lot	Yes, limited a little	No, not limited at all
a. Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Lifting or carrying groceries	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Climbing several flights of stairs?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
e. Climbing one flight of stairs?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
f. Bending, kneeling, or stooping?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
g. Walking more than a mile?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
h. Walking several blocks?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
i. Walking one block?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
j. Bathing or dressing yourself?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

3. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	No, none of the time	Yes, a little of the time	Yes, some of the time	Yes, most of the time	Yes, all of the time
a. Cut down the amount of time you spent on work or other activities	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Accomplished less than you would like	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Were limited on the kind of work or other activities	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Had difficulty performing ht work or other activities (for example, it took extra effort)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious).

	No, none of the time	Yes, a little of the time	Yes, some of the time	Yes, most of the time	Yes, all of the time
a. Cut down the amount of time you spent on work or other activities	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Accomplished less than you would like	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Didn't do work or other activities as carefully as usual	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

5. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

☐ Not at all ☐ Slightly ☐ Moderately ☐ Quite a bit ☐ Extremely

6. How much bodily pain have you had during the past 4 weeks?

☐ None ☐ Very mild ☐ Mild ☐ Moderate ☐ Severe ☐ Very Severe

7. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and house work)?

☐ Not at all ☐ A little bit ☐ Moderately ☐ Quite a bit ☐ Extremely

8. These questions are about how much you feel and how things have been with you during the past 4 weeks. For each question, please give one answer that comes closest to the way you have been feeling.

How much of the time <u>during the past 4 weeks:</u>	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of pep?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Have you been a very nervous person?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Have you felt so down in the dumps that nothing could cheer you up?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. Have you felt calm and peaceful?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
e. Did you have a lot of energy?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
f. Have you felt downhearted and blue?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
g. Did you feel worn out?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
h. Have you been a happy person?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
i. Did you feel tired?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

9. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (Like visiting friends, relatives, etc.)?

☐ All of the time
 ☐ Most of the time
 ☐ Some of the time
 ☐ A little of the time
 ☐ None of the time

10. Please choose the answer that best describes how true or false each of the following statements is for you.

	Definitely True	Mostly True	Not Sure	Mostly False	Definitely False
a. I seem to get sick a little easier than other people	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. I am as healthy as anybody I know	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. I expect my health to get worse	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d. My health is excellent	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

11. Please mark the appropriate box with an X to indicate how would you rate your own quality of life during the past four weeks

Lowest quality applies to someone completely dependent physically on others, seriously troubled mentally, unaware of surroundings and in a hopeless position.

Highest quality applies to someone physically and mentally independent, communicating well with others, able to do most of the things enjoyed, pulling own weight, with a hopeful yet realistic attitude.

Lowest → ☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9 ☐ 10 ← Highest Quality

Now, we'd like to ask you some questions about your physical health may have changed.

12. Compared to one year ago, how would you rate your physical health in general now?

☐ Much better
 ☐ Somewhat better
 ☐ About the same
 ☐ Somewhat worse
 ☐ Much worse

13. Compared to one year ago, how would you rate your emotional problems (such as feeling anxious, depressed, or irritable) now?

☐ Much better
 ☐ Somewhat better
 ☐ About the same
 ☐ Somewhat worse
 ☐ Much worse

APPENDIX VI

AUA GU SYMPTOM SCORING SCALE

Patient I.D. Sticker:

Please circle your score below.

1. Over the last month or so, how many times did you most typically get up to urinate from the time you went to bed at night until the time you got up in the morning?

☐ None ☐ 1 time ☐ 2 times ☐ 3 times ☐ 4 times ☐ 5 or more times

2. Over the past month or so, how often have you had a sensation of not emptying your bladder completely after you finished urinating?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

3. Over the past month or so, how often have you had to urinate again less than two hours after you finished urinating?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

4. Over the past month or so, how often have you found that you stopped and started again several times when you urinated?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

5. Over the past month or so, how often have you found it difficult to postpone urination?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

6. Over the past month or so, how often have you had a weak urinary stream?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

7. Over the past month or so, how often have you had to push or strain to begin urination?

☐ None ☐ Less than 1 time in 5 ☐ Less than half of time ☐ About half the time ☐ More than half the time ☐ Almost always

TOTAL SCORE: ____/35

APPENDIX VII

END OF STUDY BIOPSY REPORTING FORM

Patient I.D. Sticker

1. Date of Procedure: / /

If procedure was refused, enter date of refusal

2. Prostate Diagram

Outline the hypoechoic Transrectal Ultrasound findings. Place an X at the location of each biopsy site

3. Ultrasound Probe Characteristics

MHZ of probe .

4. Ultrasound Sizing: All measurements should be made to obtain maximum dimension

Prostate Size

a. Widths (axial plane): . cm

b. Antero-posterior: . cm

c. Length (longitudinal): . cm

(continued on next page)

5. Summary of Findings:

Region #	Echogenicity			DRE Results		Biopsied?		Extension through capsule		Seminal vesicle invasion	
	hypo	iso	hyper	nmI	abnl	yes	no	yes	no	yes	no
1	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
2	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
3	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
4	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
5	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
6	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
7	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
10	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
11	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		
12	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>		

General Comments:

- Record results of the DRE done at the time of TRUS with biopsy. If DRE was not done at this time, please indicate in General Comments.
- Record the seminal vesicle invasion as determined by TRUS only.

APPENDIX XIII

PILL DIARY • U.C. DAVIS CANCER CENTER • RADIATION ONCOLOGY DEPT

Patient's Name: _____

Instructions for the Patient: This is a monthly calendar on which you are to record the number of pills you are taking. Be sure you have enough calendars to last until your next appointment. If you develop any side effects from the pill, mark this on the calendar on the day you note the effect. **Bring the bottle(s) with the unused pills and your calendars with you each time you have an appointment.**

If you have any questions, contact: _____ Telephone: _____

Your next appointment is: _____

SPECIAL INSTRUCTIONS: _____

MONTH: _____

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY

Patient Signature _____

Date _____

Section to be completed by the nurse or research associate. Review the pill diary and check for toxicities. Report adverse reactions and toxicities according to protocol instructions. Complete the items below and update the specific Flow Sheet.

Report period: Start date: ____/____/____ End date: ____/____/____ Total pills taken this month _____
(mm/dd/yy) (mm/dd/yy) based on pill count

COMMENTS: _____

Signature: _____

Date: _____

APPENDIX IX

VITAMIN D PATIENT HANDOUT

Following is information about the 'Regular' Vitamin D adapted from the Federal Government's National Institutes of Health Information Web-Page [<http://www.cc.nih.gov/ccc/supplements/vitd.html>]:

- **Vitamin D: What is it?**

Vitamin D, calciferol, is a fat-soluble vitamin. It is found in food, but also can be made in your body after exposure to ultraviolet rays from the sun.^{1,2} Vitamin D exists in several forms, each with a different activity. Some forms are relatively inactive in the body and have limited ability to function as a vitamin. The liver and kidney help convert vitamin D to its active hormone form.³

- The major biologic function of vitamin D is to maintain normal blood levels of calcium and phosphorus.⁴ Vitamin D aids in the absorption of calcium, helping to form and maintain strong bones. It promotes calcium absorption by working with a number of other vitamins, minerals, and hormones. Without vitamin D, bones can become thin, brittle, soft, or misshapen. Vitamin D prevents rickets in children and osteomalacia in adults, which are skeletal diseases that result in defects that weaken bones.^{5,6}

What are the sources of vitamin D?

- ***Food sources***

Fortified foods are the major dietary sources of vitamin D. Prior to the fortification of milk products in the 1930s, rickets (a bone disease seen in children) was a major public health problem in the United States. Milk in the United States is fortified with 10 micrograms (400 IU) of vitamin D per quart, and rickets is now uncommon in the U.S.⁷

- One cup of vitamin D-fortified milk supplies about one-fourth of the estimated daily need of this vitamin for adults. Although milk is fortified with vitamin D, dairy products made from milk such as cheese, yogurt, and ice cream are generally not fortified with vitamin D. Only a few foods naturally contain significant amounts of vitamin D, including fatty fish and fish oils. The table of selected food sources of vitamin D suggests dietary sources of vitamin D.

- ***Exposure to sunlight***

Exposure to sunlight is an important source of vitamin D. Ultraviolet (UV) rays from sunlight trigger vitamin D synthesis in the skin.⁸ Season, latitude, time of day, cloud cover, smog, and sunscreens affect UV ray exposure. For example, in Boston the average amount of sunlight is insufficient to produce significant vitamin D synthesis in the skin from November through February. Sunscreens with a sun protection factor of 8 or greater will block UV rays that produce vitamin D, but it is still important to routinely use sunscreen whenever sun exposure is longer than 10 to 15 minutes. It is especially important for individuals with limited sun exposure to include good sources of vitamin D in their diet.

Is there a Recommended Dietary Allowance for vitamin D for adults?

- The *Recommended Dietary Allowance (RDA)* is the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in each life-stage and gender group. There is insufficient evidence to establish a RDA for vitamin D. Instead, an Adequate Intake (AI) level of intake sufficient to maintain healthy blood levels of an active form of vitamin D has been established. The 1998 AIs for vitamin D for adults, in micrograms (mcg) and International Units (IUs) are: [only those relevant for the study population is indicated below]:

Life-Stage	Men
• Ages 51-69	• 10 mcg* or 400 IU
• Ages 70 +	• 15 mcg* or 600 IU

- *1 mcg vitamin D = 40 International Units (IU)

• *Vitamin D and cancer*

Laboratory, animal, and epidemiologic evidence suggests that vitamin D may protect against some cancers. Some dietary surveys have associated increased intake of dairy foods with decreased incidence of colon cancer.⁹⁻¹¹ Another dietary survey associated a higher calcium and vitamin D intake with a lower incidence of colon cancer.¹² Well-designed clinical trials need to be conducted to determine whether vitamin D deficiency increases cancer risk, or if an increased intake of vitamin D protects against some cancers. Until such trials are conducted, it is too early to advise anyone to take vitamin D supplements to prevent cancer.

What is the health risk of too much vitamin D?

There is a high health risk associated with consuming too much vitamin D.¹³ Vitamin D toxicity can cause nausea, vomiting, poor appetite, constipation, weakness, and weight loss.¹⁴ It can also raise blood levels of calcium, causing mental changes such as confusion. High blood levels of calcium also can cause heart rhythm to change. The unhealthy collection of calcium and phosphate in soft tissues (called "calcinosis") like the kidney can be caused by vitamin D side effects.

- Consuming too much vitamin D through diet alone is not likely unless you routinely consume large amounts of cod liver oil. It is much more likely to occur from high intakes of vitamin D in supplements. The Food and Nutrition Board of the Institute of Medicine considers an intake of 25 mcg (1,000 IU) for infants up to 12 months of age and 50 mcg (2,000 IU) for children, adults, pregnant, and lactating women to be the tolerable upper intake level (UL). A daily intake above the UL increases the risk of bad health effects and is not advised.

REFERENCES FOR VITAMIN D HANDOUT

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2. Reichel H, Koeffler H, Norman AW. The role of vitamin D endocrine system in health and disease. *N Engl J Med* 1989;320:980-991.
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APPENDIX X**TISSUE SAMPLE CONSENT FORM**
CONSENT FOR USE OF SPECIMENS FOR
FUTURE RESEARCH PURPOSES
(tissue, blood, urine and other body materials)**CONSENT TO USE OF SPECIMENS FOR RESEARCH**
UNIVERSITY OF CALIFORNIA, DAVIS*page 1 of 4*

Investigator's Name(s): Srinivasan Vijayakumar, M.D., Ralph W. deVere White, M.D., Samir Narayan, M.D., Janice K. Ryu, M.D., Paul Gumerlock, M.D., Laurel Beckett, Ph.D., Ralph Green, M.D.

Department: Radiation Oncology, Urology, Hematology & Oncology, Epidemiology & Preventive Medicine, Pathology

Telephone Number(s): (916) 734-7888, (916) 734-3604 Emergency Phone: (916) 734-2011

Using Specimens for Research Purposes

At the time of your surgery or biopsy, a small piece of tissue was removed for diagnosis. We would like to keep some of the tissue that is left for **future** research purposes. If you agree, these **specimen(s)** will be kept and used to learn more about your disease as well as other diseases.

The research that may be done with your specimen(s) probably will not benefit you directly nor have an effect on your care, nor will it prevent you from participating in other research. It might help people who have your disease and other diseases in the future. Any reports about the research, done with your specimen(s), will not be shared with you or your doctor and the reports will not be put in your health record. No identifying information such as your name, address or phone number will be indicated in any research report.

Things to Think About

The decision to let us keep the specimen(s) for research purposes is up to you. No matter what you decide to do, it will not affect your care.

Even if you have already consented to let us use your specimen(s), you can change your mind at any time. Just let us know that you do not want us to use your specimen(s) and it will no longer be used.

page 2 of 4

Sometimes tissue/blood/urine are used for genetic research (about diseases that are passed on in families). Even if your tissue or blood is used for this kind of research, the results will not be put in your health records.

Your specimen(s) will only be used for research purposes. The research done with your specimen(s) may help to develop new products in the future. Please be aware that you will not have any property rights or ownership interests in products or data which may be derived from the use of your specimen(s).

Benefits

The benefits of research using specimens include learning more about what causes diseases, how to prevent them, how to treat them, and how to cure them.

Risks

There are very few risks to you. The greatest risk is the release of information from your health records which may be necessary for us to obtain along with your specimens. We will protect your records so that your name, address, and phone number will be kept private.

Where Do Specimens Come From?

Generally, a specimen may be from a blood sample, urine, or from bone marrow, skin, toenails or other body materials (in this study, the biopsy specimen will come from your prostate). People who are trained to handle specimens and protect donors' rights make sure that the highest standards of quality control are followed.

Why Do People Do Research With Specimens?

Research with specimens can help to find out more about diseases, how to prevent them, how to treat them, and how to cure them.

What Type of Research Will Be Done With My Specimen?

Many different kinds of studies use specimens. Some researchers may develop new tests to find diseases. Others may develop new ways to treat and even cure diseases. In the future, some of the research may help to develop new products, such as tests and drugs. Some research looks at diseases that are passed on in families (called genetic research). Research done with your specimen may look for genetic causes and signs of disease.

page 3 of 4

Will I Find Out the Results of the Research Using My Specimen?

You will not receive the results of research done with your specimen. This is because research can take a long time and must use specimen samples from many people before results are known. Results from research using your specimen may not be ready for many years and will not affect your care right now, but they may be helpful to people like you in the future.

Why Do You Need Information From My Health Records?

In order to do research with your specimen, researchers may need to know some things about you. (For example: are you male or female? What is your race or ethnic group? How old are you? Have you ever smoked?) This helps researchers answer questions about diseases. The information that will be given to the researcher may include your age, sex, race, diagnosis, treatments, and family history. This information is collected by your hospital from your health record.

Will My Name Be Attached to the Records That Are Given to the Researcher?

No, you will remain anonymous. Your sample will be identified by a case number, which can be linked to your personal information (your name, disease classifications, ethnic status, family history), which will be kept in a secure data bank with our statistician.

How could the Records Be Used in Ways That Might Be Harmful To Me?

Sometimes, health records have been used against patients and their families. For example, insurance companies may deny a patient insurance or employers may not hire someone with a certain illness (such as AIDS or cancer). The results of genetic research may not apply only to you, but to your family members too. For disease caused by gene changes, the information in one person's health record could be used against family members.

How Am I Protected?

Your name, address, phone number and any other identifying information will be taken off anything associated with your specimen before it is given to the researcher. Your tissue will be stored by case number. This case number can be linked to your personal information, which is kept in a secure Data Bank with our Clinical Research Associates.

What If I Have More Questions?

If you have any questions, please talk to the research investigator who provided you this form.

page 4 of 4

CONSENT

Your signature below will indicate that you will allow us to use your specimens(s) for future research purposes. You will be given a signed and dated copy of this form to keep.

Signature of Donor _____ **Date** _____

Signature of Principal Investigator _____ **Date** _____

APPENDIX XI

DOD Vitamin D5
UC Davis – Department of Radiation Oncology
ELIGIBILITY CRITERIA - CHECKLIST

Subject Initials: _____

Subject Number: _____

- ☐ Subject has completed radiotherapy with curative intent within 5 years from the date of registration, but not within the immediate twelve months.
Radiotherapy could have been external beam RT [XRT] alone, XRT with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], brachytherapy alone, brachytherapy with neoadjuvant hormonal therapy of brief duration [not exceeding 12 months], or a combination of XRT and brachytherapy [again, if neoadjuvant hormonal therapy was given, it should have been for a duration not exceeding 12 months]
- ☐ Subject had Digital Rectal Examination and documentation of the pre-RT findings in a AJCC Staging Sheet.
- ☐ Subject had Pre-treatment biopsy with pathology report of Gleason Sum.
- ☐ Subject had documented non-metastatic prostate cancer, i.e., no clinical or imaging evidence of distant metastases or lymph-node metastases.
- ☐ Pre treatment PSA level is between 2 and 8
- ☐ PSA has been stable [no more than 0.75 ng/ml variation in the PSA value], with at least 3 measurements within 12 months prior to the date of registration. (PSA doubling time must be ≤ 6 months)
- ☐ Subject is classified as Group II or III based on T-stage, Gleason Sum and PSA criteria:
- (not eligible) Group I = T1/T2 AND Gleason Sum <6 AND PSA < 10 ng/ml
- ☐ Group II = One of the three factors higher than under Group I
- ☐ Group III = Two or more of the three factors higher than under Group I
- ☐ Subject has no evidence of metastatic disease at the time of registration.
- ☐ Subject is not currently on Androgen Deprivation Therapy.
- ☐ Subject is not currently on and has not used 5- α reductase inhibitor, such as Proscar, within the last 12 months.
- ☐ Subject Karnofsky Performance Status [KPS] is $\geq 80\%$.
- ☐ Subject has no simultaneous or second malignancies within 5 years of registration.
- ☐ Subject did not undergo prostatectomy as part of treatment for prostate cancer or other conditions
- ☐ Subject has signed and been given a copy of the informed consent form.
- ☐ Subject is ≥ 18 years of age. (There is no maximum age limit for study subjects.)
- ☐ Subject has no future plans to father children.
- ☐ Subject is able to swallow and retain oral medicine.

Study Coordinator _____

Date _____

APPENDIX XII

DOD Vitamin D5 Initial Visit Form
University of California Davis
Department of Radiation Oncology

Subject Initials _____

Subject Number _____

Instructions: Complete this form at the appropriate follow-up visit and whenever there is a change in the patient's status.
Use-0 for unknown or not applicable unless otherwise specified in the code table.

<p>1 <u> </u>/<u> </u>/<u> </u> Date of Assessment</p> <p>2 <input type="checkbox"/> Quality of Life Form Complete 1 Not Completed 2 Completed</p> <p>3 <input type="checkbox"/> AUA GU Symptom Scale Complete 1 Not Completed 2 Completed</p> <p>4 <input type="checkbox"/><input type="checkbox"/><input type="checkbox"/> Karnofsky Performance Status (9= unknown)</p> <p>5 <input type="checkbox"/> Digital Rectal Examination 0. Not Done/Unknown 1. No Palpable Disease 2. Palpable Disease</p> <p>6 <input type="checkbox"/> + <input type="checkbox"/> = <input type="checkbox"/> Pre-treatment Gleason Score</p> <p>7 Pre-Treatment TNM Stage</p> <div style="display: flex; justify-content: space-around; margin-top: 10px;"> <div style="border: 1px solid black; padding: 5px; text-align: center; width: 40px;">T</div> <div style="border: 1px solid black; padding: 5px; text-align: center; width: 40px;">N</div> <div style="border: 1px solid black; padding: 5px; text-align: center; width: 40px;">M</div> </div>	<p>8 Baseline Laboratory Values 1 Not Done 2 Normal 3 Abnormal 4 Unknown</p> <p><input type="checkbox"/> Chemistry Panel Date <u> </u>/<u> </u>/<u> </u></p> <table border="1" style="width: 100%; text-align: center; margin-top: 5px;"> <tr> <td style="width: 25%;">Na</td> <td style="width: 25%;">Cl</td> <td style="width: 25%;">BUN</td> <td style="width: 25%;">Glu</td> </tr> <tr> <td>K</td> <td>HCO₃</td> <td>Cr</td> <td></td> </tr> </table> <p><input type="checkbox"/> Calcium, Magnesium, Phosphate Date <u> </u>/<u> </u>/<u> </u></p> <table border="1" style="width: 100%; text-align: center; margin-top: 5px;"> <tr> <td style="width: 33%;">Ca</td> <td style="width: 33%;">Mg</td> <td style="width: 33%;">PO₄</td> </tr> </table> <p><input type="checkbox"/> Albumin Date <u> </u>/<u> </u>/<u> </u></p> <div style="border: 1px solid black; padding: 2px; text-align: center; margin-top: 5px; width: 60px;">Alb</div> <p><input type="checkbox"/> PTH Date <u> </u>/<u> </u>/<u> </u></p> <div style="border: 1px solid black; padding: 2px; text-align: center; margin-top: 5px; width: 60px;">PTH</div> <p><input type="checkbox"/> Urine Electrolytes Date <u> </u>/<u> </u>/<u> </u></p> <table border="1" style="width: 100%; text-align: center; margin-top: 5px;"> <tr> <td style="width: 20%;">uNa</td> <td style="width: 20%;">uK</td> <td style="width: 20%;">uCl</td> <td style="width: 20%;">uCr</td> <td style="width: 20%;">uO₂</td> </tr> </table>	Na	Cl	BUN	Glu	K	HCO ₃	Cr		Ca	Mg	PO ₄	uNa	uK	uCl	uCr	uO ₂
Na	Cl	BUN	Glu														
K	HCO ₃	Cr															
Ca	Mg	PO ₄															
uNa	uK	uCl	uCr	uO ₂													

APPENDIX XII

9 Current Medications

- 1 _____
- 2 _____
- 3 _____
- 4 _____
- 5 _____
- 6 _____
- 7 _____
- 8 _____
- 9 _____
- 10 _____
- 11 _____
- 12 _____

10 Additional Treatments Since Completion of
Radation Therapy

- ☐ 1 No (Skip to end of form)
- ☐ 2 Yes (Complete form)
- 9 Unknown

☐ Additional Therapy For Prostate Cancer or
Complications of Initial Prostate Cancer
Treatment
Specify _____

☐ Additional Therapy For Prostate or Other
Genitourinary Conditions/Treatments
Specify _____

☐ Additional Medications or Therapies (For
Any Condition) Since Last Follow-up Visit
Specify _____

11 Comments _____

Signature

Date

APPENDIX XIII

DOD Vitamin D5 Follow-Up Visit Form
University of California Davis
Department of Radiation Oncology

Subject Initials _____

Subject Number _____

Instructions: Complete this form at the appropriate follow-up visit and whenever there is a change in the patient's status.
Use-0 for unknown or not applicable unless otherwise specified in the code table.

1 ___/___/___ Date of Assessment

2 ☐ Quality of Life Form Complete
1 Not Completed
2 Completed

3 ☐ AUA GU Symptom Scale Complete
1 Not Completed
2 Completed

4 ☐ ☐ ☐ Karnofsky Performance Status
(9= unknown)

5 ☐ Digital Rectal Examination
0. Not Done/Unknown
1. No Palpable Disease
2. Palpable Disease

6 ☐ ☐ ☐ Weight

7 ☐ ☐ ☐ Pill Count (Count remaining pills)

8 Baseline Laboratory Values

- 1 Not Done
2 Normal
3 Abnormal
4 Unknown

☐ Chemistry Panel

Date ___/___/___

Na	Cl	BUN	Glu
K	HCO3	Cr	

☐ Calcium, Magnesium, Phosphate

Date ___/___/___

Ca	Mg	PO4
----	----	-----

☐ Albumin

Date ___/___/___

Alb

☐ PTH

Date ___/___/___

PTH

☐ Urine Electrolytes

Date ___/___/___

uNa	uK	uCl	uCr	uCa
-----	----	-----	-----	-----

APPENDIX XIII

9 Complications of Treatment (Record date of 1st appearance. Use 0=absent and 1=present. If reaction is severe please give a description)

☐ Asymptomatic Hypercalcemia _/_/_/

☐ Hot Flashes _/_/_/

☐ Nausea _/_/_/

☐ Emesis _/_/_/

☐ Diarrhea _/_/_/

☐ Abdominal Pain _/_/_/

☐ Loss of Appetite _/_/_/

☐ Renal Calculi _/_/_/

☐ Bone Pain _/_/_/

☐ Other Gastrointestinal
Specify _____ _/_/_/

☐ Other Genitourinary
Specify _____ _/_/_/

☐ Hematologic
Specify _____ _/_/_/

☐ Dermatologic
Specify _____ _/_/_/

☐ Cardiovascular
Specify _____ _/_/_/

☐ Other
Specify _____ _/_/_/

10 Additional Treatments Since Last Follow-up Visit

- ☐ 1 No (Skip to end of form)
☐ 2 Yes (Complete form)
☐ 9 Unknown

☐ Additional Therapy For Prostate Cancer or
Complications of Initial Prostate Cancer
Treatment
Specify _____

☐ Additional Therapy For Prostate or Other
Genitourinary Conditions/Treatments
Specify _____

☐ Additional Medications or Therapies (For
Any Condition) Since Last Follow-up Visit
Specify _____

11 Comments _____

Signature _____

Date _____

DOD Vitamin D5 Telephone Contact Form
University of California Davis
Department of Radiation Oncology

Subject Initials _____

Subject Number _____

Instructions: Complete this form at the appropriate follow-up visit and whenever there is a change in the patient's status.
Use 0 for unknown or not applicable unless otherwise specified in the code table.

<p>1 Complications of Treatment (Record date of 1st appearance. Use 0=absent and 1=present. If reaction is severe please give a description)</p> <p><input type="checkbox"/> Asymptomatic Hypercalcemia ____/____/____</p> <p><input type="checkbox"/> Hot Flashes ____/____/____</p> <p><input type="checkbox"/> Nausea ____/____/____</p> <p><input type="checkbox"/> Emesis ____/____/____</p> <p><input type="checkbox"/> Diarrhea ____/____/____</p> <p><input type="checkbox"/> Abdominal Pain ____/____/____</p> <p><input type="checkbox"/> Loss of Appetite ____/____/____</p> <p><input type="checkbox"/> Renal Calculi ____/____/____</p> <p><input type="checkbox"/> Bone Pain ____/____/____</p> <p><input type="checkbox"/> Other Gastrointestinal Specify _____ ____/____/____</p> <p><input type="checkbox"/> Other Genitourinary Specify _____ ____/____/____</p> <p><input type="checkbox"/> Hematologic Specify _____ ____/____/____</p> <p><input type="checkbox"/> Dermatologic Specify _____ ____/____/____</p> <p><input type="checkbox"/> Cardiovascular Specify _____ ____/____/____</p> <p><input type="checkbox"/> Other Specify _____ ____/____/____</p>	<p>2 Additional Treatments Since Last Follow-up Visit</p> <p><input type="checkbox"/> 1 No (Skip to end of form)</p> <p><input type="checkbox"/> 2 Yes (Complete form)</p> <p><input type="checkbox"/> 9 Unknown</p> <p><input type="checkbox"/> Additional Therapy For Prostate Cancer or Complications of Initial Prostate Cancer Treatment Specify _____</p> <p><input type="checkbox"/> Additional Therapy For Prostate or Other Genitourinary Conditions/Treatments Specify _____</p> <p><input type="checkbox"/> Additional Medications or Therapies (For Any Condition) Since Last Follow-up Visit Specify _____</p> <p>3 Comments _____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>_____</p> <p>Signature _____</p> <p>Date _____</p>
---	---

DOD Vitamin D5 Study Completion Form
University of California Davis
Department of Radiation Oncology

Subject Initials _____

Subject Number _____

Instructions: Complete this form at the appropriate follow-up visit and whenever there is a change in the patient's status.
Use-0 for unknown or not applicable unless otherwise specified in the code table.

1 / / Date of Assessment2 ☐ Quality of Life Form Complete
1 Not Completed
2 Completed3 ☐ AUA GU Symptom Scale Complete
1 Not Completed
2 Completed4 Karnofsky Performance Status
(9= unknown)5 ☐ Digital Rectal Examination
0. Not Done/Unknown
1. No Palpable Disease
2. Palpable Disease6 Weight7 Pill Count (Count remaining pills)
** Please collect all Study Medications

8 Final Laboratory Values

- 1 Not Done
- 2 Normal
- 3 Abnormal
- 4 Unknown

☐ Chemistry PanelDate / /

Na	Cl	BUN	Glu
K	HCO3	Cr	

☐ Calcium, Magnesium, PhosphateDate / /

Ca	Mg	PO4
----	----	-----

☐ AlbuminDate / / ☐ PTHDate / / ☐ Urine ElectrolytesDate / /

uNa	uP	uCl	uO	uCa
-----	----	-----	----	-----

9 Complications of Treatment (Record date of 1st appearance. Use 0=absent and 1=present. If reaction is severe please give a description)

☐ Asymptomatic Hypercalcemia _/_/_

☐ Hot Flashes _/_/_

☐ Nausea _/_/_

☐ Emesis _/_/_

☐ Diarrhea _/_/_

☐ Abdominal Pain _/_/_

☐ Loss of Appetite _/_/_

☐ Renal Calculi _/_/_

☐ Bone Pain _/_/_

☐ Other Gastrointestinal
Specify _____

☐ Other Genitourinary
Specify _____

☐ Hematologic
Specify _____

☐ Dermatologic
Specify _____

☐ Cardiovascular
Specify _____

☐ Other
Specify _____

10 Additional Treatments Since Last Follow-up Visit

- ☐ 1 No (Skip to end of form)
2 Yes (Complete form)
9 Unknown

☐ Additional Therapy For Prostate Cancer or Complications of Initial Prostate Cancer Treatment
Specify _____

☐ Additional Therapy For Prostate or Other Genitourinary Conditions/Treatments
Specify _____

☐ Additional Medications or Therapies (For Any Condition) Since Last Follow-up Visit
Specify _____

12 End of Study Biopsy Completed

- ☐ 1 No
2 Yes
9 Unknown

Biopsy Findings _____

11 Comments _____

Signature

Date

CONSENT TO OPERATION, PROCEDURES, BLOOD TRANSFUSION AND ANESTHESIA

The purpose of this form is to advise you of important information regarding the operation or procedure(s) that your doctor has recommended to you. **PLEASE READ THE ENTIRE FORM CAREFULLY BEFORE SIGNING IT.**

I authorize _____, M.D., and those who he/she may designate as associates or assistants to perform the following operation or medical procedure _____

_____ as well as any related or incidental diagnostic or therapeutic procedures that they believe may be necessary.

I understand that I will be informed of any substitution of the doctor named above and will be given the opportunity to refuse substitution.

I ACKNOWLEDGE THAT THE FOLLOWING INFORMATION HAS BEEN EXPLAINED TO ME:

- (a) The purpose and expected benefits of the proposed operation or procedure described above;
- (b) significant risks or possible complications that are known to be associated with it;
- (c) reasonable alternative methods of treatment (if any);
- (d) The possible effects to my health if I should refuse to undergo the operation or procedure; and
- (e) research or economic interests (if any) that are related to the performance of this operation or procedure.

BLOOD TRANSFUSIONS

I understand that unless a medical emergency exists, or it was determined to be medically inadvisable, my doctor will have informed me if there was a reasonable possibility that a transfusion or blood or blood components may be necessary. I understand that there are various options available to me regarding blood transfusion, including the right to refuse blood or blood components. I understand that refusing transfusions that are recommended by my doctor(s) may result in life-threatening consequences to me.

I understand that certain risks and complications may be associated with blood transfusions, including, but not limited to transmission of infectious diseases and transfusion reaction.

ANESTHESIA

I authorize the administration of anesthesia if it is determined to be necessary to assure my safety and comfort. I understand that certain risks and complications may be associated with anesthesia use and that they have been discussed with me, as well as reasonable alternative choices of anesthesia (if any).

AUTHORIZATION AND CONSENT:

By my signature below, I confirm that:

- (1) I have read this form;
- (2) I have been given the opportunity to discuss with my doctor(s) any questions that I may have regarding the nature and purpose of this operation or procedure, and my questions have been answered fully and to my satisfaction;
- (3) I understand that the operation or procedure may not accomplish the desired purpose and that no promises or guarantees of any kind have been made to me as to the result or cure; and
- (4) I understand that extra services such as laboratory studies or x-rays may be ordered if determined by my doctor(s) to be necessary;
- (5) I have the right to consent or to refuse any proposed operation or procedure prior to its performance.

PATHOLOGY SERVICES

I authorize the hospital pathologist, at his or her discretion, to retain, preserve, or dispose of any tissues, organs or medical devices that may be removed during the procedure subject to the following conditions (if any),

NO INFORMATION REQUESTED:

Although given the opportunity to have this information explained to me, I specifically decline to be advised of the nature, benefit, risks and alternatives to the proposed operation/procedure as well as those associated w/anesthesia.

Date _____

PATIENT OR PATIENT'S LEGAL REPRESENTATIVE AND RELATIONSHIP
OF REP. TO THE PATIENT

Time _____

INFORMANT AND PRINTED NAME OF INFORMANT

CV of Dr. Rachel Chou, Medical Monitor for the Study

Curriculum Vitae

RACHEL H. CHOU, M.D.

Assistant Professor
Co-Director of Radiosurgery Program
University Of California Davis Cancer Center
4501 X Street, G-126
Sacramento, CA 95817
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EDUCATION

Wellesley College, Wellesley, MA – Magnum Cum Laude	B.A. in Chemistry	1984-1988
Tufts University School of Medicine, Boston, MA	M.D.	1988-1992

POST GRADUATE TRAINING AND EXPERIENCE

Internal Medicine Intern, St. Mary's Hospital/Medical Center, San Francisco, CA	1992-1993
Radiation Oncology Resident, University of California San Francisco, San Francisco, CA - Chief Resident, Radiation Oncology, 1995-1996	1993-1996
Staff Radiation Oncologist, CHEM Center for Radiation Oncology, Stoneham, MA	1996-1998
Assistant Clinical Professor, Department of Radiation Oncology, University of California Davis, Sacramento, CA	1998-1999
Associate, Duke University Medical Center, Durham, NC	1999-2000
Clinical Affiliate, Moore Regional Hospital, Pinehurst, NC	2001-2002
Staff Physician, Craven Regional Medical Center, New Bern, NC	2001-2002
Assistant Professor, University of California, Davis Medical Center, Sacramento, CA	2002-present
Intensity Modulated Radiation Therapy Training Course	2003/June
Gamma Knife Training Course, Stockholm	2003/June

FELLOWSHIPS, HONORS, AND AWARDS

Freshman Distinction and Dean's List, Wellesley College	1984-1988
Zimmerman Scholarship and Fellowship, Wellesley College	1986
Phi Beta Kappa, Wellesley College	1987

RACHEL H. CHOU, M.D.
11/19/2003

Page 2

Sigma Xi, Wellesley College	1987-1988
Senior Honors Thesis, Wellesley College	1987-1988
Honors in Radiation Oncology, Tufts University	1988

RESEARCH EXPERIENCE

Comparison and Categorization of Contemporary Chinese Short Stories, Wellesley College	1986
Fatty Acid Composition of Three Marine Unicellular Cyanobacteria, Wellesley College	1986-1987
P-31 NMR Spectroscopy of Cultured Human Ocular Melanoma Cells, Honors Thesis, Massachusetts Institute of Technology-Massachusetts Eye and Ear Infirmary	1987-1988
Toxicities of Total Body Irradiation and Chemotherapy for Pediatric Bone Marrow Transplantation, University of California, San Francisco	1993-1994
Radiotherapy for AIDS-CNS Lymphoma, University of California, San Francisco	1995-1996
Role of Oral Glutamine in Preventing Radiation-Induced Stomatitis in Patients with Head and Neck Cancer, CHEM Center, Stoneham, MA, Lahey-Hitchcock Medical Center, Burlington, MA	1997-1998
Acute Toxicities and Sexual Dysfunction Following Three Dimensional Conformal Radiation Therapy for Prostate Cancer, University of California, Davis	1998-1999
Role of Oral Glutamine in Preventing Radiation-Induced Stomatitis in Patients with Head and Neck Cancer, Duke University Medical Center, Durham, NC	1999-2001
Acute Toxicities of Prostate Cancer Patients Treated with Brachytherapy, Duke University Medical Center, Durham, NC	1999-2001
Clinical Applications of Intensity Modulated Radiation Therapy for Head and Neck Cancer	2002-present
Radiosurgery/Gamma Knife Program, University of California, Davis, Sacramento, CA	2003-present

RESEARCH FUNDING

US Bioscience – Co-Investigator: A Phase II Trial of Subcutaneous Amifostine and Radiation Therapy in Patients with Head and Neck Cancer, \$43,000, Duke University Medical Center	1999
Amgen - Co-Investigator: A Phase II Study of rHuKGF in Head and Neck Cancer, \$45,920, Duke University Medical Center	1999-2000
Bristol Myers Squibb – Principle Investigator: A Pilot Study of Oral Glutamine in Head and Neck Cancer Patients Receiving Concomitant Chemotherapy with	2000-2001

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Hyperfractionated Radiotherapy, \$42,800, Duke University Medical Center

Pharmacia Upjohn – Co-Investigator: A Phase I/II Study of Irinotecan and Whole Brain Radiation Therapy in Patients with Brain Metastases from Solid Tumors, \$216,000, UC Davis Medical Center

2003-present

TEACHING/PRESENTATION

P-31 NMR Spectroscopy of cultured human ocular melanoma cells, Wellesley College Senior Honors Thesis Presentation, Wellesley, MA	1988
Total body irradiation, Nursing Education and Research, Department of Nursing, University of California, San Francisco	1994
The acute and delayed toxicities of total body irradiation for pediatric bone marrow transplantation, International Society of Pediatric Oncology Annual Meeting, Paris, France	1994
Total body irradiation and pediatric bone marrow transplantation, Nursing Education and Research, Department of Nursing, University of California, San Francisco	1995
Basic concepts of cancer chemotherapy and hormonal therapy, Radiation Oncology Technology Student Education, Radiation Oncology Technology Department, City College of San Francisco	1995
Ductal carcinoma In Situ of the breast – A Radiation Oncologist's perspective, Melrose Wakefield Hospital Grand Rounds, Melrose, MA	1997
Early invasive breast cancer and DCIS for women in the 90's, CHEM Center, Staff Lecture Series, Stoneham, MA	1997
Carcinoma of the nasopharynx: An overview and recent advances, Duke University Grand Rounds, Durham, NC	1999
Acute toxicity of three-dimensional conformal radiotherapy in prostate cancer patients eligible for implant monotherapy, Radiological Society of North America Annual Meeting, Chicago, IL	1999
Radiation oncology resident head and neck review course, Duke University, Durham, NC	2000
Introduction of head and neck malignancy for rotating medical students and oncology fellows, University of California, Davis, Sacramento, CA	2002-present
Challenging cases in lung cancer, panelist for panel discussion, Network for Oncology Communications and Research, San Diego, CA	2003
The Use of Amifostine as a Radioprotectant in Head and Neck Cancer Otolaryngology Journal Club, University of California, Davis/Kaiser Permanente, Sacramento, CA	2003

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Co-Moderator for Medical Oncology Journal Club, University of California, Davis,
Sacramento, CA

March 2003

Radioprotection and Amifostine in Head and Neck Cancer, Department of
Radiation Oncology, University of California, Davis, Sacramento, CA

2003

PUBLICATIONS

Merritt, M.V., S.P. Rosenstein, C. Loh, **R.H. Chou**, and M.M. Allen. A comparison of the major lipid classes and fatty acid composition of marine unicellular cyanobacteria with freshwater species. *Archives of Microbiology* 155(2):107-113, 1991.

Chou, R.H., G.B. Wong, J.H. Kramer, D.W. Wara, K.K. Matthay, M.R. Crittenden, P.S. Swift, M.J. Cowan and W.M. Wara. Toxicities of total-body irradiation for pediatric bone marrow transplantation. *Int. J. Radiat. Oncol. Biol. Phys* 34(4):843-851, 1996.

Chou, R.H., R.B. Wilder, M. Ji, J.K. Ryu, B.R. Leigh, J.D. Earle, R.L.S. Doggett, H.D. Kubo, M. Roach III and R.W. deVere White. Acute toxicity of three-dimensional conformal radiotherapy in prostate cancer patients eligible for implant monotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 47(1):115-119, 2000.

Wilder, R.B., **R.H. Chou**, J.K. Ryu, R.L. Stern, M.S. Wong, M. Ji, M. Roach, III and R. deVere White. Potency preservation after three-dimensional conformal radiotherapy for prostate cancer. *Amer. J. Clin. Oncol.* 23(4):330-333, 2000.

Kang, S.K., **R.H. Chou**, R.K. Dodge, R.W. Clough, H-S.L. Kang, M.G. Bowen, B.A. Steffey, S.K. Das, S-M. Zhou, A.W. Whitehurst, N.J. Buckley, J.H. Kim, R.E. Joyner, I. Sarmina, G.S. Montana, S.S. Ingram and M.S. Anscher. Acute urinary toxicity following transperineal prostate brachytherapy using a modified Quimby loading method. *Int. J. Radiat. Oncol. Biol. Phys.* 50(4):937-945, 2001

Chou, R.H., R.B. Wilder, M.S. Wong and K.M. Forster. Recent advances in radiotherapy for head and neck cancers. *Ear, Nose & Throat Journal* 80(10):704-719, 2001

Kang, S.K., **R.H. Chou**, R.K. Dodge, R.W. Clough, H.L. Kang, C.A. Hahn, A.W. Whitehurst, N.J. Buckley, J.H. Kim, R.E. Joyner, G.S. Montana, S.S. Ingram and M.S. Anscher. Gastrointestinal toxicity of transperineal interstitial prostate brachytherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 53(1):99-103, 2002

IN PRESS

A.Y. Chen, **R.H. Chou**, S.J. Shih, D. Lau, D. Gandara. Enhancement of Radiotherapy with DNA Topoisomerase I-Targeted Drugs, *Critical Review Oncology Hematology*, 2003.

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IN PREPARATION

R.H. Chou, A.Y. Chen, D. Lau. Promising Role of Irinotecan for Treatment of Brain Metastases. *Lancet Oncol*, 2003.

BOOK CHAPERS

Chou, R.H., C.G. Lee and M.S. Anscher. Chapter 7: Radiation therapy for disease of the biliary tree and gallbladder, in *Disease of the Gallbladder and Bile Ducts: Diagnosis and Treatment*, edited by P.A. Clavien and J.M. Baillie, Blackwell Science, Inc., Malden, MA, 2001.

Chou, R.H. and R.B. Wilder. The role of radiation therapy in treatment of head and neck malignancies in head and neck cancer. *Management of the difficult case*, 2nd ed., edited by P.J. Donald, Thieme Medical Publishers, New York, In Press.

ABSTRACTS

Chou, R.H., G.B. Wong, J.H. Kramer, D.W. Wara, K.K. Matthay, M.J. Cowan and W.M. Wara. The acute and delayed toxicities of total body irradiation (TBI) for pediatric bone marrow transplantation (BMT). *Medical and Pediatric Oncology* 23(3):185, 1994.

Chou, R.H., G.B. Wong, P.S. Swift, J.H. Kramer, D.W. Wara, K.K. Matthay, M.J. Cowan and W.M. Wara. Toxicities of total body irradiation (TBI) for pediatric neuroblastoma bone marrow transplantation (BMT). *Proceedings of the American Society of Clinical Oncology* 14:447, 1995.

Chou, R.H., R.B. Wilder, M. Ji, J.K. Ryu, B.R. Leigh, J.D. Earle, H.D. Kubo, M. Roach and R. deVere White. Acute toxicity of 3D prostate cancer patients eligible for implant monotherapy. *Proceedings of the Radiologic Society of North America* 230:153, 1999.

Kang, S.K., **R.H. Chou**, R.K. Dodge, R. W. Clough, M.G. Brown, B.A. Steffey, S.K. Das, S.M. Zhou, A.W. Whitehurst, N.J. Buckley, J.H. Kim, R.E. Joyner, I. Sarmina and M.S. Anscher. Acute urinary toxicity following transperineal prostate brachytherapy. *Proceedings of the American Society for Therapeutic Radiology and Oncology* 48(3S):253, 2000.

Chou, R.H., S.K. Kang, R.K. Dodge, R.W. Clough, H.L. Kang, C.A. Hahn, A.W. Whitehurst, N.J. Buckley, J.H. Kim, R.E. Joyner, G.S. Montana, S.S. Ingram and M.S. Anscher. Acute gastrointestinal toxicity of transperineal interstitial prostate brachytherapy. *Proceedings of the American Radium Society*, 2001.

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SPECIAL INTERESTS/EXPERTISE

Three-Dimensional Conformal Treatment Planning

Intensity Modulated Radiation Therapy

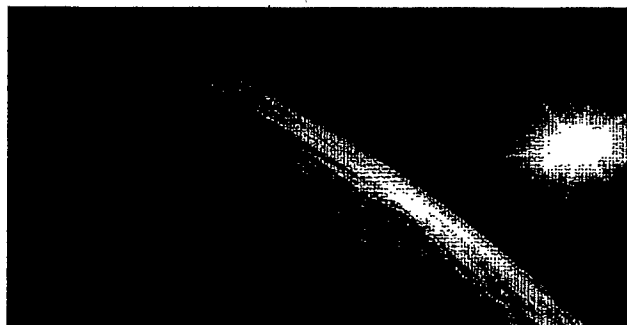
Stereotactic Radiosurgery

REFERENCES

Available upon request.

INFORMED CONSENT FORM

Cover Sheet

FAX**OFFICE OF HUMAN
RESEARCH PROTECTION**Date March 10, 2004Number of pages including cover sheet 8

To: Srinivasan Vijayakumar, MD
C/o Phil Boerner

Dept: Radiation Oncology

FAX: 4-7076

From: Mihaela Harris
OHRP Analyst

Phone: 916-734-6865

FAX: 916-734-6872

REMARKS: ☒ Urgent ☒ For your review ☐ Reply ASAP ☐ Please Comment

PLEASE DELIVER TO ADDRESSEE AS SOON AS POSSIBLE

NOTE TO INVESTIGATOR: Please follow the instructions detailed in the attached letter of action when submitting your response to the IRB.

1) First address all IRB concerns in a cover memorandum, restating each IRB concern in a numbered list followed by your response. 2) Revise the appropriate document(s), if requested to do so, and **bold** and underline the changes on the revised documents. Please number each of these revisions, in the margin of the document(s), concordant with the list on the cover memo so as to facilitate their identification. 3) Provide at least one clean copy of the revised document(s) without the bold and underlines so that this copy can be stamped with IRB approval and used as your copy to present to subjects. 4) Attach a copy of this Notice and reviewer's comments on top of your response.

Please Note: Do not use the Modification/Amendment Form to respond to IRB concerns/revisions. Please address your response in a memorandum and attach the revised documents. The Modification/Amendment Form is utilized after IRB approval of a new or continuing study and during the term of approval.

UNIVERSITY OF CALIFORNIA, DAVIS

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SANTA BARBARA · SANTA CRUZ

OFFICE OF THE VICE CHANCELLOR FOR RESEARCH
916 734-6864
FAX: 916 734-6872

SACRAMENTO, CALIFORNIA 95817

March 9, 2004

Srinivasan Vijayakumar, MD
MED RADIATION ONCOLOGY
c/o Phil Boerner
Fax: 916-734-7076

RE: Protocol #: 200412214-1 "A Phase I/II Double-Blind, Randomized Clinical Trial To Prevent/Delay Biochemical And Clinical Failure In High-Risk, Non-Metastatic Prostate Cancer Patients After Radiotherapy, Using 1a-Hydroxyvitamin D5 Versus Placebo: A Tolerance-Finding And Intermediate Biomarker Response-Seeking Study"

Committee Meeting Date: 03/08/04

Dear Colleague:

The Institutional Review Board (IRB) has conducted their review of the above referenced study. In order to quickly communicate to you the concerns of the IRB, we are providing you the direct comments of the reviewer(s) (see attached). Since Committee members prepare their comments in advance of a meeting, there will be instances in which issues or concerns noted on the form are resolved during committee discussions. In these instances, the issues have been "lined out" and do not need to be addressed due to their resolution in Committee. Should the IRB Reviewers refer you to our Model Consent Form or other forms, they will be attached to this letter for your use. Principal Investigators are reminded that until approval documents have been issued, no new study may begin nor a continuing study continue. Federal, State and University regulations make no provision for any grace period extending the conduct of an existing research study, beyond the expiration date of IRB approval. Instructions for resubmission to the IRB are indicated below.

Instructions for Resubmission to the IRB: 1) First address all IRB concerns in a cover memorandum, restating each IRB concern in a numbered list followed by your response. 2) Revise the appropriate document(s), if requested to do so, and **bold** and underline the changes on the revised documents. Please number each of these revisions, in the margin of the document(s), concordant with the list on the cover memo so as to facilitate their identification. 3) Provide at least one clean copy of the revised document(s) **without** the bold and underlines so that this copy can be stamped with IRB approval and used as your copy to present to subjects. 4) Attach a copy of this Notice on top of your response and submit the following number of copies:

Approved Pending Minor Revisions:

Submit 2 copies of your response
to the OHRP at the Ambulatory Care Center,
Suite 3870, UCDCMC. Once reviewed and
approved, approval documents will be
issued to the principal investigator.

Should you have any questions regarding this notification, please contact the Analyst to this Committee, Mihaela Harris at (916) 734-6865.

Sincerely,

Barth Wilsey, MD
Chair, Institutional Review Board

INSTITUTIONAL REVIEW BOARD

University of California, Davis
Office of Human Research Protection

IRB Primary Reviewer ☐

IRB Secondary Reviewer X

IRB Member ☐

IRB Chair ☐

Meeting Date: 03-08-2004

Protocol No. 200412214-1

PI Name: S. Vijayakumar M.D.

Please indicate the specific information investigators need to provide or the changes they need to make, in language that can be transmitted directly to the investigator. You must make sure to provide the **REASON** for a specific change or concern. All comments must be printed - no abbreviations. Identify the documents as follows:

CV - Cover Page(s)	DOS - Description of Study	IDF - Investigational Drug Form	AF - Assent Form	CF - Consent Form(s)
IDV - Investigational Device Form	Q - Questionnaire(s)	DA - Drug Addendum	S - Survey	ADV - Advertisement
BOR - Bill of Rights	PR - Progress Report	LRP- Letter to Research Participant		

SUMMARY OF THE STUDY (the Primary and Secondary Reviewer are required to note for the official IRB minutes. This requirement also applies to studies Approved As Submitted):

This is a Phase I/II trial of 1alpha-hydroxyvitamin D5 vs placebo taken orally by high-risk, non-metastatic prostate cancer patients after treatment with radiotherapy (at least 1 year after but no more than 5 years after treatment) for the primary disease. There will be 40 patients (20 on study med and 20 on placebo) taking medication for 2 years. The study hopes to evaluate the safety of the drug and it's effectiveness in chemoprevention of recurrent disease. This study is sponsored by the Department of Defense.

Document	Page	Heading	Paragraph	Reason for Concern/Revision	Address/Revise As Follows
DOS	1	Purpose	1	This is a phase I/II safety/chemoprevention study to determine whether taking a non-toxic Vitamin D analog	Eliminate wording "non-toxic" as toxicity not totally established yet.
CF	11		4 - starting: For the first month....	"13 cc"	Change to acceptable medical abbreviation of: 13 ml
				"3 tablespoons"	13 ml is equal to about 3 teaspoons or 1 tablespoon
			1	"13 cc"	Same as above.
	12			"3 tablespoons"	

INSTITUTIONAL REVIEW BOARD

INSTITUTIONAL REVIEW BOARD

University of California, Davis
Office of the Vice Chancellor for Research

IRB Chairperson Meeting Date: 3/8/2004
 REVIEWER'S PROPOSED CHANGES
 OHRP IRB Guidebook
 UC Davis OHRP Link
 Protocol No. 200412214-1
 PI Name Vijayakumar
 FDA
 UC Davis HIPAA

Please indicate the specific information investigators need to provide or the changes they need to make, in language that can be transmitted directly to the investigator. **You must make sure to provide the REASON for a specific change or concern. All comments must be printed - no abbreviations.** Identify the document as follows:

CV - cover page(s) DOS - Description of Study IDF - Investigational Drug Form AF - Assent Form
 CF - Consent Form(s) IDV - Investigational Device Form Q - Questionnaire(s)
 DA - Drug Addendum S - Survey ADV - Advertisement BOR - Bill of Rights PR - Progress Report

Document	Page	Heading	Paragraph	Reason for Concern/Revision	Concern/Revision
			No Issue	NEW STUDY Summary of the background and study design/objectives:	Overall description of study Phase I and II study of a Vitamin D analogue, 1-alpha hydroxyvitamin D5 for prostate CA recurrence Forty randomized patients will receive either D5 or placebo, 12-60 months after completion of RT Laboratory and prostate biopsy results will be tallied The Purpose and Background are written clearly with the following items noted: Adequate statement of the research problem Specific aims of study are clearly stated Procedures are consistent with sound research design Researcher is qualified to conduct study PI disclosure of financial conflict of interest The Methods are written clearly with the following items noted: Clear description of all activities involving human subjects Total duration of study clear and appropriate, frequency of activities is described Number of subjects and duration of participation is stated and appropriate Detailed summary of data to be collected (e.g. prostate biopsy samples, lab values, etc) Plan to monitor data to ensure subject safety (e.g. Calcium levels, etc.) You state, in the consent, that the biopsy "will not cost you any additional expense" State that the sponsor will pay for the biopsy if that is the case
Cf DOS				Discuss whether or not the biopsy is part of standard care in the consent and DOS	Followed clearly for in 4-5 Nucle
				Please provide a sample size determination for your study.	When the sample size is too small to yield valid conclusions or an hypothesis is imprecisely formulated, subjects may be exposed to risk without sufficient justification. While good research design may not itself reduce or eradicate risks to subjects, poor or faulty research design means that the risks are not likely to be reasonable in relation to the benefits. To help assess the research design, a biostatistician may be consulted if the need arises. Not all procedures designed to increase the statistical validity of a study may be justified. Procedures, even those included for purposes

					of good research design, that add disproportionate risks to subjects may be unacceptable. INDs and IDEs
			No Issue	IND obtained	<p>Clinical trials conducted under an IND or IDE issued by FDA are obligated to adhere to the protocol as submitted. Any modification, such as extension to another age group, use of a different dose, change in subject eligibility criteria, must be approved by FDA as well as the IRB prior to implementation, unless immediate action is required to eliminate apparent immediate hazards to human subjects.</p> <p>Federal law prohibits the distribution of new drugs, biologics, and medical devices until FDA has reviewed clinical data and determined that a particular product is safe and effective for a specific use in human patients. In order to test a new drug, biologic, or device in clinical trials, it is necessary to obtain an exemption from that law. Thus a drug or device sponsor is required to apply for an Investigational New Drug exemption (IND) or an Investigational Device Exemption (IDE) before tests with human subjects may begin. In general, the review requirements for biologics are the same as those for drugs. Accordingly, unless otherwise indicated, the provisions that follow use the term 'drug' to apply to drugs as well as biologics. The investigator is responsible for obtaining the IND or IDE number and providing it to the IRB.</p> <p>If an investigator is the developer of the drug or device and no commercial manufacturer is involved, then either the investigator or the investigator's institution may be the sponsor for purposes of designing and organizing clinical trials. The sponsor is responsible for submitting an IND or IDE application to FDA and providing a copy of the FDA's response to the OHRP. Sponsors also have important administrative and reporting requirements above and beyond those of investigators. Faculty contemplating the dual role of sponsor-investigator should consult with OPRs staff about the additional responsibilities that entails.</p> <p>The IND or IDE application must contain sufficient data from animal and in vitro studies to demonstrate the likelihood that the product will be safe and effective for the purpose indicated. If the FDA agrees that the data are sufficient to support a decision to initiate clinical trials, and that the proposed protocol is acceptable, FDA will provide an IND or IDE number for the protocol.</p> <p>The investigator is required to wait for 30 days after FDA receives the IND or IDE application, to permit FDA scientists to review the materials and, if necessary, request additional information, require modifications, or disapprove the application. FDA notifies the sponsor of the date it receives the IND. The OHRP will not provide formal approval for a study until the 30 days have elapsed and FDA has either provided an IND or IDE number or advised the investigator that an IND or IDE is not required [21 CFR 312.40 (b)] (drugs) and [21 CFR 812.30 (a)] (devices).</p>
			No Issue	Data safety monitoring and adverse event reporting addressed in the IXOS	<p>Please add a Data Safety Monitoring Plan to the DOS. Suggested verbiage (if applicable): "This is a single site study and the DSMP will consist of adverse event reporting. There will not be a need for an interim analysis nor DSMB". Alternative suggested verbiage: "This multisite study will have a DSMB with interim analysis. Stopping rules are being formulated".</p> <p>Explain your reporting mechanism for reporting adverse and serious adverse events to the IRB. Suggested verbiage: "We will report adverse events that are serious, unanticipated and related within 5 working days to the IRB. These adverse events will also be reported to the sponsor and oversight body [NIH, FDA, GCRC, RAC where applicable] within the designate reporting interval. We will report all adverse events (serious or otherwise, on or off-site) at the time of the continuing review of this study. An exception to the last sentence applies if a DSMB is involved. One may substitute, 'A DSMB report will be tendered in lieu of a table of adverse events from all sites at the time of Continuing Review of this study'.</p> <p>This information should be placed in your Description of Study:</p>

					<p>DESCRIPTION OF STUDY:</p> <p>1. PURPOSE, METHODS, AND PROCEDURES: describe in detail the purpose, research methods and procedures of the study. Address how you will monitor this study to ensure that the study is being conducted according to the protocol. Clarify whether you will have a Data Safety Monitoring Board to conduct such monitoring.</p> <p>2.</p> <p>3. RISKS: describe any potential risks to subjects, physical, psychological, social or legal. Assess the likelihood and seriousness of those risks. If the methods of research create potential risks, describe other methods, if any, that were considered and why they will not be used. Address procedures for maintaining confidentiality if confident it represents a risk. Explain your reporting mechanism for reporting adverse and serious adverse events to the IRB.</p> <p>.....</p>
					<p>Please use the MODEL CONSENT FORM FOR USE OF SPECIMENS FOR FUTURE RESEARCH PURPOSES [issue, blood, urine and other body materials] found on the Human Subjects webpage hyperlink http://over.ucdavis.edu/Forms/hr71%5a5.doc</p> <p>The following information is provided in this model consent form.</p> <p>Using Specimens for Research Purposes:</p> <p>Things to Think About</p> <p>Benefits</p> <p>Risks</p> <p>Where Do Specimens Come From?</p> <p>Why Do People Do Research With Specimens?</p> <p>What Type of Research Will Be Done With My Specimen?</p> <p>Will I Find Out the Results of the Research Using My Specimen?</p> <p>Why Do You Need Information From My Health Records?</p> <p>Will My Name Be Attached to the Records That Are Given to the Researcher?</p> <p>How Could the Records Be Used in Ways That Might Be Harmful to Me?</p> <p>How Am I Protected?</p> <p>Health Insurance Portability and Accountability Act (HIPAA)</p> <p>What If I Have More Questions?</p> <p>Signature</p> <p>Please note: The form is currently in anonymized format. If you are going to disclose identifiers, then the 'Will My Name Be Attached to the Records That Are Given to the Researcher?' section must be changed to reflect this fact. In addition, please delete the 'How Am I Protected?' section and just using the HIPAA section to set forth the disclosures.</p> <p>The Risk Section is written clearly with the following items noted:</p> <p>Risks are minimized by using sound design</p> <p>Unnecessary risks are excluded</p> <p>Where applicable, use of placebo is appropriate</p> <p>PI will monitor for hypercalcemia</p> <p>The process for monitoring and reporting adverse events is adequate</p> <p>Adequate provisions exist for protecting subject privacy</p>
					<p>A. Educator and Clinician in Vitro Fertilization</p> <p>B. Women</p> <p>C. Children and Minors</p> <p>D. Cognitively Impaired Persons</p> <p>E. Terminally Ill Patients</p> <p>F. Elderly/Aged Persons</p> <p>G. Minorities</p> <p>H. Students, Employees, and Normal Volunteers</p>
					<p>OHRP model sample consent form will be used</p> <p>No issue</p> <p>Risks to participation (physical, psychological, social, legal):</p> <p>Vulnerable Population, if any: none</p>
					<p>No issue</p> <p>No issue</p>

						<p>D. Cognitively Impaired Persons E. Prisoners F. Traumatized and Comatose Patients</p> <p>J. Students, Employees, and Normal Volunteers K. International Research</p> <p>Subpart A – Federal Policy for the Protection of Human Subjects (Basic DHHS) Policy for Protection of Human Research Subjects) Subpart B – Additional Protections for Pregnant Women, Human Fetuses and Neonates Involved in Research Subpart C – Additional DHHS Protections Pertaining to Biomedical and Behavioral Research Involving Prisoners as Subjects Subpart D – Additional DHHS Protections for Children Involved as Subjects in Research</p>
		<p>No Issue</p> <p><input checked="" type="checkbox"/> HIPAA applies <input type="checkbox"/> Not a HIPAA study because it is epidemiological in nature</p>	<p>The only studies covered by HIPAA are: 1) those in which data is obtained from a covered entity (such as UC/DHS); or 2) those in which health care provision is part of the study and the health care is provided in a setting where the provider normally bills for care (e.g., a clinical trial at UC/DHS).</p>	<p>Protected health information is being used to recruit subjects per the HIPAA Worksheet PI has: a) addressed how they plan to protect the identifiers used to identify potential subjects. b) addressed how and when they plan on destroying these identifiers c) provided written assurance to the IRB that no identifiers, used for recruitment purposes, will be disclosed to a third party except as required by law or for authorized oversight of the research project.</p>	<p>"Conditional" Approvals. Convened IRBs often set conditions under which a protocol can be approved. OHRP recommends the following guidelines in such cases: (a) When the convened IRB requests substantive clarifications or modifications regarding the protocol or informed consent documents that are directly relevant to the determinations required by the IRB under HHS regulations at 45 CFR 46.111, IRB approval of the proposed research should be deferred, pending subsequent review by the convened IRB of responsive material. (b) Only when the convened IRB stipulates specific revisions requiring simple concurrence by the investigator may the IRB Chair or another IRB member designated by the Chair subsequently approve the revised research protocol on behalf of the IRB under an expedited review procedure. Your reply (if not approved as submitted):</p> <p><input type="checkbox"/> Approved as submitted <input checked="" type="checkbox"/> Committee recommends Conditional Approval (provide 2 copies)</p>	<p><input type="checkbox"/> Approval Deferred. Please provide 24 copies for evaluation by full Committee</p> <p>[1] First address all of IRB concerns in a cover memorandum restating each IRB concern in a numbered list followed by your response [2] Revise the appropriate document(s) if requested to do so, and bold and underline the changes on the revised documents. Please number each of these revisions in the margin of the document(s) concordant with the list on the cover memo so as to facilitate their identification [3] Provide at least one clean copy of the revised document(s) so that this copy can be stamped with IRB approval and used as your copy to present to subjects [4] Attach a copy of all action letters by Committee members on top of your response and submit the correct number of copies (see attached resubmission form)</p>